EVALUATION OF BACTERIAL POLYMERS AS PROTECTIVE AGENTS FOR SENSITIVE PROBIOTIC BACTERIA

By

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DECLARATION

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ABSTRACT

Probiotics are live microorganisms which when administered in adequate amounts confer one or more health benefits on the host. Different processing conditions, the acidic condition of the stomach and exposure to hydrolytic enzymes affect the viability and efficacy of probiotic organisms. This study investigated the protective effects of two biopolymers poly-gamma-glutamic acid (γ-PGA) and bacterial cellulose (BC) on probiotics during freeze drying and during exposure to simulated intestinal juices and bile salts. The antibacterial property of *Bifidobacterium* strains was also investigated against four pathogenic bacteria. γ-PGA, a naturally occurring biopolymer was produced by two bacteria (*Bacillus subtilis* ATCC 15245 and *B. licheniformis* ATCC 9945a) in GS and E media, γ-PGA yields of about 14.11g/l were achieved in shake flasks and molecular weight of up to 1620 k Da was recorded, γ-PGA production was scaled up in a fermenter with *B. subtilis* using GS medium. BC, an edible biopolymer was produced by *Gluconacetobacter xylinus* ATCC 23770 in HS medium and a modified HS (MHS) medium. A yield of about 1.37g/l was recorded and BC production with MHS medium was used for probiotic application. *B. longum* NCIMB 8809 *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 showed the best antimicrobial properties against the investigated pathogens. Survival of *Bifidobacterium* strains was improved when protected with powdered BC (PBC) although γ-PGA offered better protection than PBC. Viability of *B. longum* NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 in simulated gastric juice (SGJ) and simulated intestinal juice with bile salts was improved when protected with 5% γ-PGA and 5% γ-PGA+PBC with a reduction of <1 Log CFU/ml while a reduction of ≤2 Log CFU/ml was recorded in PBC protected cells. Protecting *Bifidobacterium* strains with γ-PGA, PBC or a novel γ-PGA + PBC combination is a promising method to deliver probiotic bacteria to the target site in order to confer their health benefits on the host.

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Acoustic Transducer Diaphragms	ACDs
Acquired Immune Deficiency Syndrome	AIDS
Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Bacterial cellulose	BC
Bacteria cellulose synthase	*bcs*
Bacterial vaginosis	BV
Bifidobacterium Selective Medium	BSM
Bifid triple viable capsule	BIFICO
Cellobiohydrolases	CBH
Cell Free Culture Supernatants	CFCS
Chronic Periodontitis	CP
Colony forming units	CFU
Corn steep liquor	CSL
De Man Rogosa and Sharpe	MRS
Dilution factor	Df
Dispersity index	DI
Distention, bloating and flatulence	DBF
Dry-fabricated bio-film	DFBF
Dry weight	W_d
Enterohaemorrhagic *Escherichia coli*	EHEC
Food and Agriculture Organization of the United Nations	FAO
Fourier Transform Infrared Spectroscopy	FT-IR
Fructooligosaccharide	FOS

Gastrointestinal tract	GIT
Gel permeation chromatography	GPC
Glutamate dehydrogenase	GDH
Helicobacter pylori	HP
Hestrin and Schramm	HS
High Performance Liquid Chromatography	HPLC
Human Immunodeficiency Virus	HIV
Human papillomavirus	HPV
Irritable bowel syndrome	IBS
Lactic acid bacteria	LAB
Magnetite nanoparticles	MNPs
Modified Hestrin and Schramm	MHS
Molecular number	M_n
Molecular weight	M_w
National Collection of Industrial and Marine Bacteria	NCIMB
Nuclear Magnetic Resonance Spectroscopy	NMR
Open Reading Frame	ORF
Paclitaxel poliglumex	PPX
Phosphate Buffer Saline	PBS
Polyelectrolyte complex	PEC
Polyglutamate Synthetase Complex	PgsBCA
Polyglutamic acid	PGA
Poly-alpha-glutamic acid	α-PGA
Poly-gamma-glutamic acid	γ-PGA
Poly-γ-D-glutamate	PDGA

Poly-γ-L-glutamate	PLGA
Poly-γ-L-D-glutamate	PLDGA
Polyvinyl alcohol	PVA
Polyvinylidene Filter	PVDF
Powdered bacterial cellulose	PBC
Refractive Index	RI
Scaling and root planning	SRP
Scanning Electron Microscopy	SEM
Simulated intestinal juice	SIJ
Simulated gastric juice	SGJ
Skimmed Milk Powder	SMP
Standard Error	SE
Tricarboxylic acid	TCA
Trypticase-phytone-yeast extract	TPY
Tryptone soy agar	TSA
Tryptone soy broth	TSB
World Health Organization	WHO
Uridine diphosphate glucose	UDPG
Ulcerative Colitis	UC
Urinary Tract Infections	UTIs
Water retention values	WRV
Wet weight	W_w
X-Ray Diffraction	XRD

BACKGROUND STUDY

A major cause of morbidity and mortality in the general populace is food borne diseases. The vulnerable groups such as infants, elderly and immunocompromised are highly susceptible to infections with greater likelihood of more severe illnesses, including death, and increased potential for illness with an opportunistic pathogen (Tauxe *et al*., 2010; Nyenje and Ndip, 2013).

Foods that have been contaminated with harmful bacteria, viruses, parasites or chemicals are responsible for over 200 diseases ranging from diarrhoea to cancers (WHO, 2015a).

The WHO reported that about 1 in 10 people in the world fall ill after consumption of contaminated foods (an estimate of 600 million people), while the deaths of about 2 million people including children have been linked to consumption of unsafe foods (WHO, 2015b).

Diarrhoeal diseases that result from consuming contaminated food are the major cause of illnesses and death in developing countries; about 550 million people fall ill annually and about 230,000 deaths are recorded annually. This is not limited to developing countries alone; in the United States, an estimated 76 million illnesses which result in 325,000 hospitalizations and 5000 deaths were due to foodborne diseases (Gandi and Chikindas, 2007; Ifeadike *et al*., 2015).

Food borne diseases are usually infectious or toxic in nature and are caused by viruses, bacteria, parasites or chemical substances entering the body through contaminated food or water. Food borne pathogens can cause severe diarrhoea or debilitating infections such as meningitis (WHO, 2015b).

The list of causative agents of intestinal diseases has grown over the years due to changes in food processing and eating habits as well as recognition of new microbes. *Salmonella spp*.,

Shigella spp., *Clostridium botulinum* and *Staphylococcus aureus* were recognised before 1960 as major causes of gastrointestinal diseases; *Clostridium perfringens*, and *Bacillus cereus* were added to the list in the 1960s and then rotavirus and norovirus were recognised in the 1970s. A series of additions that include *Campylobacter*, *Yersinia*, *Listeria monocytogenes*, new strains of *Escherichia coli* such as O157:H7, *Cryptosporidia* and *Cyclospora* were added in the 1980s and 1990s (Newell *et al*, 2010).

Foods such as eggs, poultry and products of animal origin are involved in the outbreak of salmonellosis. Drinking water, raw milk, raw or undercooked poultry are causes of foodborne cases with *Campylobacter*. *Enterohaemorrhagic E. coli* (EHEC) is associated with unpasteurised milk, undercooked meat and fresh fruits and vegetables. Milk and dairy products such as cheese, meat products such as beef, pork, fermented sausages, fresh produce such as radishes, cabbage, seafood and fish products have all been associated with *Listeria* contamination. The elderly, pregnant, new-born and immunocompromised populations are more susceptible to listeriosis (Gandi and Chikindas, 2007; Parada *et al*. 2007, WHO, 2015b).

Consequently, illnesses caused by consumption of contaminated foods have an immense economic and public health impact worldwide as they prevent socioeconomic development by putting a strain on the health care systems and also affect national economies, tourism and trade (Gandi and Chikindas, 2007; WHO, 2015b).

Reducing the level of illnesses from foodborne pathogens is a public health goal in many countries around the world and different approaches have been employed, one of such is development of new and improved methods of food preservation while treatment of infections from foodborne pathogens involves the use of antimicrobials including antibiotics (Smith-Palmer, 1998; Luber, 2009).

However, the last decade has experienced a dramatic increase in the proportion and absolute number of bacterial pathogens presenting multidrug resistance to antibacterial agents. Multidrug-resistant bacteria are currently considered as an emergent global disease and a major public health problem (Roca *et al*., 2015).

Antimicrobial resistance remains one of the major threats to modern medicine. This challenge of antimicrobial resistance and the increasing demand for safe foods with reduced chemical additives has heightened the interest in substituting antibiotics with natural products that are not harmful to the host and to the environment (WHO, 2015b).

Biopreservation is the use of non-pathogenic microorganisms and or their metabolites to improve microbiological safety and extend the shelf life of foods. Lactic acid bacteria (LAB) are very attractive for biopreservation due to their safe history of use (Parada *et al*., 2007).

The use of certain bacterial peptides as antimicrobial substances in the place of antibiotics for human application is a credible alternative. Among these bacterial peptides, bacteriocins produced by LAB have gained a wide attraction as they are active in a nanomolar range and are non-toxic. Bacteriocins are proteins or complexed proteins with antimicrobial action against other bacteria. Since bacteriocins are proteinaceous agents, they are quickly digested by proteases in the human digestive tract (Parada *et al*., 2007).

Several probiotic bacteria, including LAB have been reported to produce a variety of other antimicrobial compounds such as short-chain fatty acids, hydrogen peroxide, nitric oxide in addition to bacteriocins; these antimicrobial compounds may enhance their ability to compete with other gastrointestinal microbes and to inhibit pathogenic bacteria (Dobson, *et al*., 2012).

Probiotics have been defined by the WHO as “Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Jimenez-Pranteda *et*

al., 2012). The health benefits associated with consumption of probiotics are mostly strain specific but the most consistent evidence relates to preventing development, incidence and recurrence of diarrhoea. Probiotics have been reported to have the ability to prevent gastrointestinal infections and this is thought to be as a result of their ability to produce organic acids (such as lactic and acetic acids that have antimicrobial properties) and their ability to stimulate immune processes in the host (Tejero-Sarinena *et al*., 2012).

The ability of probiotics to inhibit the growth of several pathogens such as *Salmonella typhimurium* and *Escherichia coli* has been demonstrated in *in vitro* studies (Anand *et al*., 1981; Fooks and Gibson, 2002; Tejero-Sarinena *et al*., 2012). *Bifidobacterium* has been implicated in the control of the acidity of the large intestinal tract, its ability to hydrolyse lactulose and other indigestible complex carbohydrates into acetic and lactic acids thereby maintaining the intestinal microbial balance by hindering the growth of potential pathogens has also been reported (Kamalian *et al*., 2014). A probiotic mixture of five probiotic strains (*Lactobacillus murinus* DPC6002 and DPC6003, *Lactobacillus pentosus* DPC6004, *Lactobacillus salivarius* DPC6005 and *Pediococcus pentosaceus* DPC6006) has been reported to improve the clinical and microbiological outcome of *Salmonella* infection in pigs (Dobson *et al*., 2012).

However, for probiotics to exert their health benefits, they need to be metabolically stable and active in the product and the host. They need to be able to survive passage through the stomach and reach the intestines in large amount (Martin *et al*., 2015). For efficacy, the International Dairy Federation has suggested a minimum of 10^7 CFU/g probiotics in the product before consumption (Shih *et al*., 2013).

It has been reported that many strains of *Bifidobacterium* species do not have the ability to survive passage in the human gastrointestinal tract (GIT) due to the harmful impact of acidity

and bile concentration in the small intestines (Kamalian *et al*., 2014). Other factors that affect the viability of bifidobacteria include acidity, pH value, storage temperature, dissolved oxygen content, hydrogen peroxide and concentrations of lactic and acetic acids (Holkem *et al*., 2016; Kamalian *et al*., 2014).

Several methods have been employed to improve the survival of probiotic strains to the aforementioned adverse conditions which include appropriate selection of acid and bile resistant strains, the use of oxygen-impermeable containers, incorporation of microencapsulation and the use of cryoprotectants such as skimmed milk powder and sucrose (Anal and Singh, 2007; Jagannath *et al*., 2010).

Investigations have highlighted the use of biopolymers such as bacterial cellulose (BC) and poly-gamma-glutamic acid (γ-PGA) as probiotic protectants during freeze drying (Jagannath *et al*., 2010; Bhat *et al*., 2013).

γ-PGA is an edible, biodegradable and non-toxic biopolymer (Shih and Van, 2001). Fermented soy beans- a traditional food in Japan also known as mucilage of "natto" is a mixture of γ-PGA and fructans produced by *Bacillus natto* (Shih and Van, 2001). BC is an edible biopolymer commonly referred to as nata and an important source of dietary fibre with a distinctive characteristic of interwoven fibres of cellulose that form a mesh conformation with the cells attached to the cellulose fibres (Jagannath *et al*., 2010).

This study investigates the effect of coating selected probiotic strains with γ-PGA or BC on their survival during freeze drying and during passage through the upper and lower GIT. The effect of these probiotic strains on inhibiting growth of food pathogens will also be investigated.

1.0 INTRODUCTION TO PROBIOTICS

1.1 Introduction

One of the fastest growing areas in the global food industry is the area of functional foods and they are considered the result of a dietary strategy to reduce the incidence of illness in humankind (Patrignani *et al*., 2015). A wide variety of foods have been or will be characterised as functional with varying components, some as nutrients that affect a variety of body functions relevant to a state of well-being or to the reduction in risk of a disease. Hence, no simple universally accepted definition of functional foods exists (Roberfroid, 2011).

Functional foods have been defined simply as foods that may provide health benefits beyond basic nutrition (IFIC, 2011), and elaborately, as foods similar in appearance to conventional foods, which are consumed as part of a usual diet and have demonstrated physiological benefit and or reduce the risk of chronic disease beyond basic nutritional functions (Health Canada, 2002).

A food is termed functional if it is of benefit to one or more target functions in the body such that it is important to the state of well-being or reduces the risk of a disease. Functional foods are not drugs because they do not have therapeutic effects rather they mostly function in reducing the risk of a disease (Figueroa-Gonzalez *et al*., 2011; Roberfroid, 2011).

The functionality of functional foods is dependent on bioactive components such as probiotic bacteria which may be naturally present in the product but will require special formulations for optimum benefits (Korhonen, 2002).

Probiotics among functional foods have gained a lot of interest and they are the fundamental ingredients of fermented milk and some yoghurts (Figueroa-Gonzalez *et al*., 2011). Selecting a suitable means of delivery of probiotics to ensure they overcome the physical and chemical

barriers of the GIT is highly important in the development of probiotic foods (Bedani *et al*., 2013).

1.2 History

The consumption of fermented dairy products is not new, they are known to be consumed all around the world and are called different names in different regions. Table 1.1 shows some examples of fermented dairy products, raw material from which they are produced and their region or country of origin.

Table 1.1: Fermented dairy products, their raw materials and country of origin (Airag, 2010; Farnworth, 2005 and Acidified Milks, 1990)

Dairy Product	Raw Material	Country of origin
Labneh	Cow milk, sheep and or goat milk	Lebanon, Egypt, Jordan, Libya, Syria, Iraq
Kefir	Whole cow milk	Equador, Caucasian mountains of the former USSR
Airag/Kumys	Mare Milk	Mongolia, Russia
Lassi	Cow, buffalo, sheep or goat or goat milk	India, Pakistan
Boruga	Whole cow milk	Dominican Republic
Dahee	Fresh or recombined cow milk	Guayana
Jemid	Sheep and goat milk	Jordan
Leben	Cow milk	Morocco
Yoghurt	Cow, ship or buffalo milk	Near east area
Sour milk	Cow milk	Nicaragua

These dairy products, some of which are still consumed are known to be of therapeutic importance even before the bacteria responsible for the effects were discovered (Shortt, 1999). The souring of milk products was favoured in the middle East and Asia and were recommended for intestinal illnesses (Richards, 2017).

The term ‘Probiotic’ is a relatively new word which was derived from the Greek word ‘*pro bios’* which means ‘*for life’* it has been used over the years and it has different meanings to

different people and different definitions have been given. The term 'Probiotic' is used in relation to microorganisms that are of benefit to animals and humans (Fuller, 1995; Soccol *et al*., 2010).

Bifidobacteria were identified by Tissier in 1899 and their use for infant diarrhoea was promoted. In 1906, the concept of Probiotic: Bulgarian bacillus was proposed by Elie Metchnikoff and *Lactobacillus casei Shirota* was isolated by Minoru Shirota in 1930 (Sanders, 2010).

It is believed that the term "probiotic" was first used by Kollath in 1953 to describe organic and inorganic supplements necessary to restore health to patients suffering a form of malnutrition resulting from eating too much highly refined food (Hamilton-Miller *et al*., 2003).

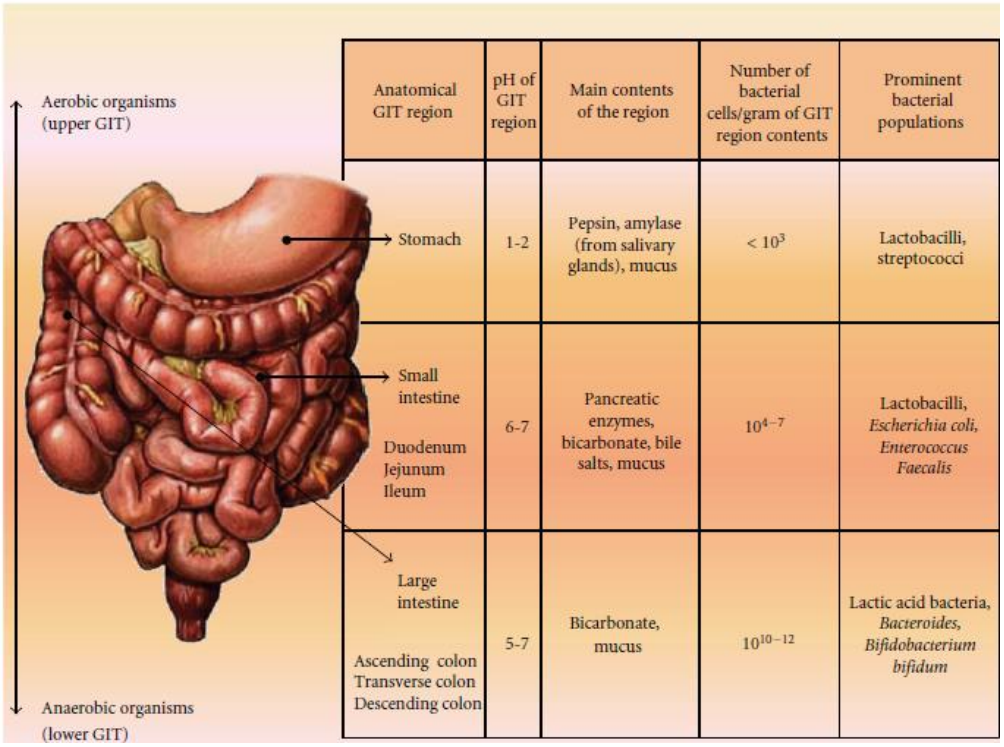
Other reports reveal that "Probiotic" was first used by Lilley and Stillwell in 1965 to describe substances that are secreted by one microorganism which stimulated the growth of another, a definition that was not accepted (Fuller, 1995).

Parker in 1974 defined Probiotics as organisms and substances which contribute to intestinal microbial balance. This definition was modified in 1989 by Roy Fuller who defined Probiotics as "*live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance"* (Fuller, 1995).

Meng *et al*., (2008); Vasiljevic and Shah, (2008) defined probiotics as living supplements or living microorganisms (bacteria or yeasts) which when ingested are able to bring about health benefits beyond basic nutrition and also are able to enhance the intestinal microbial balance of the host.

The widely-accepted definition given by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) for Probiotics is:

"*Live microorganisms (bacteria or yeast) which when administered in adequate amounts* (which is generally $>10^6$-10^8CFU/g or $>10^8$ - 10^{10}CFU/day) *confer one or more health benefits on the host"* (Anal and Singh, 2007; Aureli *et al*., 2011; Champagne *et al*., 2011). About 300 to 500 different species of bacteria are present in the human intestinal habitat; which increases in size and metabolic activity along the length of the GI tract, however, this varies between individuals and with diet. The distribution of these bacteria varies depending on the region of the GIT (Drakoularakou *et al*., 2011; Prakash *et al*., 2011) (figure 1.1).



Anatomical GIT region	pH of GIT region	Main contents of the region	Number of bacterial cells/gram of GIT region contents	Prominent bacterial populations
Stomach	1-2	Pepsin, amylase (from salivary glands), mucus	$< 10^3$	Lactobacilli, streptococci
Small intestine, Duodenum, Jejunum, Ileum	6-7	Pancreatic enzymes, bicarbonate, bile salts, mucus	10^{4-7}	Lactobacilli, *Escherichia coli*, *Enterococcus Faecalis*
Large intestine, Ascending colon, Transverse colon, Descending colon	5-7	Bicarbonate, mucus	10^{10-12}	Lactic acid bacteria, *Bacteroides*, *Bifidobacterium bifidum*

Figure 1.1 : The GIT characteristics and the localization of the gut microbiota (Adapted from Prakash *et al*., 2011)

The stomach is a highly acidic region, about 10^5 cells/g of bacteria can be isolated from the gastric contents after a meal, this value declines as the pH falls (pH 2.0- pH 3.0) which is the normal pH of stomach secretions, hence the stomach houses very low numbers of microorganisms (10^3 cells/g of bacteria) (Drakoularakou *et al*., 2011). Lactobacilli and streptococci are the predominant microorganisms in stomach and the duodenum. There is an increase in bacterial numbers in the ileum and jejunum while the jejunum and caecum are highly populated with about 10^{12} cells/g of bacteria. Organisms present in the large intestine include *Bacteroides*, lactic acid bacteria and *Bifidobacterium* species. The number and types of bacteria in the gastrointestinal tracts vary depending on environmental factors such as disease state and food intake (Prakash *et al*., 2011).

Probiotics are usually administered orally and are available in different forms such as capsules, tablets or as food products such as milk, yoghurt and yoghurt based drinks (Burgain *et al*., 2011).

1.3 Types and Examples of Probiotic Microorganisms

Several criteria must be fulfilled before a probiotic can exert health benefits, these include:

1. It must possess good technological properties for the process of incorporation into products by manufacturers
2. It should not affect the texture, taste or cause an unpleasant flavour in the finished product
3. It must be able to survive stomach acidity and enzymatic actions without losing viability and getting to its target site alive
4. It must be able to carry out its function in the gastrointestinal habitat without losing viability (Saarela *et al*., 2000).

The genera: *Bifidobacterium, Lactobacilllus* and *Streptococcus* are the most popular strains of probiotics. Some strains of *Enterococcus* and yeasts have been used as probiotics (Soccol *et al*., 2010). Table 1.2 highlights some of the lactic acid bacteria for probiotic preparations.

Table 1.2: Lactic acid bacteria commonly used in probiotic preparations (Adapted from Tripathy and Giri, 2014)

Probiotic bacteria	Species
Lactobacillus sp.	*L. acidophilus, L. casei, L. delbrueckii ssp.,* *L. cellobiosus, L. curvatus, L. fermentum, L. lactis, L. plantarum, L. reuteri, L. brevis*
Bifidobacterium sp.	*B. bifidum, B. adolescentis, B. animalis, B. infantis, B. breve B. thermophilum, B. longum*
Enterococcus sp.	*Ent. faecalis, Ent. Faecium*
Streptococcus sp.	*S. cremoris, S. salivarius, S. diacetylactis, S. intermedius*

1.4 Health Benefits of Probiotics

Probiotics are safe, economical and devoid of any long term negative side effects and have proven to be beneficial for treatment of digestive, respiratory and immunological diseases (Table 1.2). Because these organisms occur naturally in food products such as milk and yoghurt, they are widely accepted by the general populace (Prakash *et al*., 2011). It is important to emphasise that the health benefits derived from probiotics are not species or genus-specific but strain specific and no probiotic strain will provide all the proposed benefits, not even strains of the same species (Figueroa-Gonzalez *et al*., 2011).

Probiotic organisms can exert their beneficial effects on the host through any of the following five means (figure 1.2):

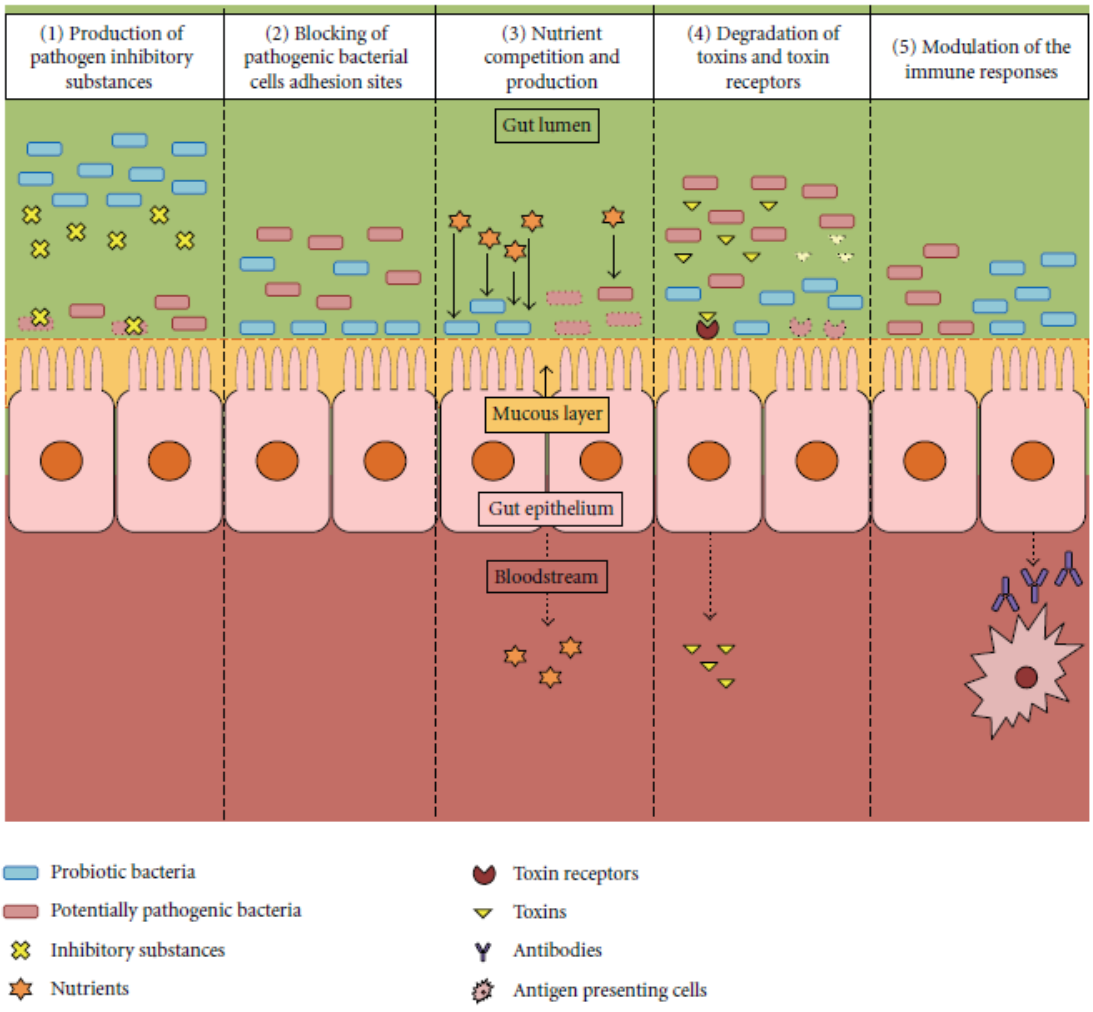


Figure 1.2 : The five means through which probiotic organisms exert their health benefits on their hosts (Prakash *et al.*, 2011).

1. **Production of pathogen inhibitory substances:** probiotic organisms are able to produce organic acids, bacteriocins or hydrogen peroxide that inhibit the growth and metabolism of pathogenic bacteria;

2. **Blocking of pathogenic bacterial cells adhesion sites:** probiotics prevent pathogenic bacteria from attaching to the intestinal epithelial surfaces by adhering to epithelial cells;

3. **Nutrient competition and production:** probiotic organisms challenge the survival of pathogenic bacteria by consumption of nutrients that would have been utilised by pathogenic bacteria;

4. **Degradation of toxins and toxin receptors:** the toxin receptors for bacteria such as *C. difficile* on the intestinal mucosa can be degraded by probiotic organisms;

5. **Modulation of the immune responses:** protection against disorders can also be through stimulation of specific and non- specific immunity by probiotic organisms. (Rolfe, 2000). Interaction of probiotic bacteria with the epithelial lining of the host attracts immune cells to the site of infection and induce specific immune markers (Ashraf and Shah, 2014). The first line of defense created by the epithelial cells in the gastrointestinal mucosa is the selectively permeable barrier between the intestinal lumen and the internal environment of the body. This barrier can be interrupted in disease conditions that cause immunological alterations resulting to inflammation of the intestinal wall and intestinal disorders, some probiotic formulations are able to prevent chronic inflammation of the GIT through the stimulation of innate immunity in the gastrointestinal epithelium (Bajagai *et al*., 2016).

The ability of probiotics to confer effects distant from the site of administration is another interesting aspect.

This can be via actual transfer of organisms, for example from gut to mammary glands of lactating women as reported by Arroyo *et al*. (2010), where women with infectious mastitis ingested either probiotic bacteria (*L. fermetnum* or *L. salivarius*) or the recommended antibiotics. They reported a lower bacterium count for the probiotic group after 21 days compared with the antibiotics group and the probiotic bacteria were isolated

from the milk samples of the probiotic group. The probiotic group also had lower recurrence of mastitis than the antibiotic group (Arroyo *et al*., 2010).

It can also be by production of molecules that are adsorbed across the intestine or that influence the host compounds directly or indirectly (Reid, 2016). Lowering of blood cholesterol is an example with mechanisms that include reduction in deoxycholic acid levels in the intestine, deconjugation of bile salt hydrolase, production of compounds that inhibit 3-hydroxy-3methylglutaryl coenzyme A and through the assimilation of cholesterol (Guo and Li, 2013; Oner *et al*., 2013; Tomaro-Duchesneau *et al*., 2014).

Reduction of the severity and duration of respiratory tracts infection by probiotic strains is another distant site effect that has been reported and this is likely to be via enhancement in immune mediators (Reid, 2016). De Vrese and co-workers (2006) reported a reduction in severity and duration of common cold, after a long-term consumption of a probiotic formulation that contained *L. gasseri*, *B. longum*, and *B. bifidum* with vitamins and minerals by healthy adults. It was also reported that there was an increase in cytotoxic T cell plus T suppressor cell count and T- helper cell counts (de Vrese *et al*., 2006).

Other health benefits of probiotic bacteria are summarised in table 1.3.

Table 1.3: Health Benefits of Probiotics

Health Area	Application	Function
Gastro intestinal disorders	Eradication of *Helicobacter pylori* (HP) infection	HP infected patients were grouped into a triple–plus-probiotics or a triple-plus only group. They were evaluated after 4 weeks of receiving treatments and it was reported that the patients that received the triple-plus-probiotics treatment showed a higher eradication rate than patients that received triple-plus only treatment. It was concluded that probiotic supplements that contain *Bacillus subtilis* and *Streptococcus faecium* have demonstrated to improve drug compliance, reduce side effects and enhance the intention-to-treat eradication rate of HP (Park *et al*., 2007).
	Ulcerative Colitis (UC)	Bifid triple viable capsule (BIFICO) or an identical placebo was administered to 30 patients with UC after treatment with UC capsule standard therapy for 8 weeks. From the results, 20% in the BIFICO group had a relapse during the follow up period compared with 93.3% in the control group who suffered a relapse. The administration of the probiotic preparation was suggested to be able to prevent flare up of chronic UC and it can also be used as a prophylactic drug to reduce cases of relapse in UC patients (Cui *et al*., 2004).
	Reduction of diarrhoeal symptoms in human immunodeficiency virus/ Acquired Immune Deficiency Syndrome (HIV/ AIDS) patients	Conventional yoghurt fermented with *L. delbruekiivar bulgaricus* and *Streptococcus thermophilus* supplemented with probiotic *L. rhamnosus* GR-1 and *L-reuteri* RC-14 was administered to 24 HIV/AIDS patients. The patients had CD4 counts over 200; they were not receiving antiretroviral or dietary supplements but showed clinical signs of moderate diarrhoea. The patients consumed 100ml/day of supplemented or unsupplemented yoghurt for 15 days. Although the CD4 count either stayed the same or increased, the diarrhoeal symptoms were eradicated in all probiotic treated subjects (12/12) within 2 days compared with 2/12 in patients that received unsupplemented yoghurt (Anukam *et al*., 2008).
	Antibiotic induced diarrhoea	Two oncology patients who developed antibiotics induced diarrhoea were treated with probiotics. Relapsing and chronic diarrhoea were resolved in both patients after probiotic treatment (Benchimol and Mack, 2004).
	Traveller's diarrhoea (TD)	A meta-analysis was carried to compare the efficacy of probiotics for the prevention of TD based on published randomised and clinical trials. It was discovered that *Saccharomyces boulardii* and a mixture of *L. acidophilus* and *B. bifidum* had significant and it was concluded that probiotics are safe and effective for the prevention of TD (McFarland, 2007)

Table 1.3 Continued

Health Area	Application	Function
Effects against infections	Prevention of post-operative infections after abdominal surgery	The effects of probiotics/synbiotics on reduction of post-operative infection was evaluated. It was reported that probiotics/synbiotics reduced surgical site infections and UTIs from abdominal surgeries compared to the control group (Lytvyna, 2016).
	Reduction of severity and duration of common cold	The effect of long term consumption of probiotic bacteria on common cold (influenza) was investigated in a double blind, randomised, controlled trial in healthy adults not vaccinated against influenza. It was discovered that long term consumption of probiotic bacteria reduced the severity of symptoms and shortened the duration of the episodes by about 2 days (de Vrese *et al*., 2006).
	Prevention of urogenital infections	A systematic review was carried out to determine the efficacy of probiotics in the prevention and treatment of urogenital tract infections in adult women. At least one species of *Lactobacillus* probiotic was used as an intervention for treatment or prevention out of the 20 cases reviewed. 14 of the cases were focused on bacterial vaginosis (BV), 3 on urinary tract infections (UTIs), 2 on vulvovaginal candidiasis and 1 on human papillomavirus (HPV). The use of probiotics was effective for the prevention and treatment of BV, clearing HPV lesions and prevention of recurrences of candidiasis and UTIs. There were no reports of adverse effects relating to the use of probiotics (Hanson *et al*., 2016).
	Treatment of UTIs in children	A search was done to investigate the efficacy of probiotics in prophylaxis and treatment of UTIs in children. It was reported that probiotics are able to inhibit uropathogens by competing for receptors and nutrients and also through immune modulation and production of inhibitory metabolites. It was concluded that probiotics may be effective as natural immune modulators for prevention and treatment of UTIs in children (Beyitler and Kavukcu, 2016).
	Control of infectious diarrhoea	The mean duration of acute diarrhoea was shortened among children in a nursery after the administration of probiotic yoghurt containing *L. casei* compared to children that consumed conventional yoghurt (Kechagia *et al*., 2013).

Table 1.3 Continued

Health Area	Application	Function
Cancer	Reduction in risk of liver cancer	Aflatoxins are a group of mycotoxins and well-known human hepatocarcinogens produced by the common fungus *Aspergillus paraciticus*. A study was carried out to investigate if the administration of probiotic bacteria could block the intestinal absorption of aflatoxin B_1 which will result in a reduction in urinary excretion of aflatoxin B_1-N^7- guanine, a marker for a biologically effective dose of aflatoxin exposure. 90 healthy men were grouped and a group was administered *L. rhamnosus* LC705 and *Propionibacterium freudenreichii subsp. Shermanii* strains 2 times daily for 5 weeks and the other group received a control preparation. Results after 5 weeks showed a significant reduction in the concentration of urinary AFB-N^7-guanine in the probiotic administered group compared to the control group. It was concluded that probiotic supplements can offer an effective dietary approach to reducing the risk of liver cancer cell (El-Nezami *et al*., 2006).
	Colon cancer	Inhibition of colon cancer by yoghurt containing lactic acid bacteria was investigated using BALB/c mice. Yoghurt was administered to the mice for 10 days; colon tumours were induced using dimethylhydrazine injection and the mice were fed with yoghurt for 6 months after induction. It was reported that tumour growth was inhibited in the yoghurt fed group compared to the control group (de Moreno de LeBlanc, 2007).
	Breast cancer	The effect of consuming *L. helveticus* R389 fermented milk or its proteolytic variant *L. helveticus* L89 on a murine hormone-dependent breast cancer model was investigated. Results showed that the administration of the fermented milk from both probiotic strains delayed or stopped development of tumour. It was concluded that antitumor immune response was stimulated by the administration of milk fermented by both probiotic strains (de Moreno de LeBlanc, 2005).
Others	Prevention of dental caries/reduction of plaque levels and gingival inflammation	The potential of probiotic *L. rhamnosus, L. reuteri* and *B. DN*-173010 to alter the colonisation of carcinogenic bacteria and prevent dental caries have been reported. Reduction in plaque levels and gingival inflammation have also been reported with the application of *L. reuteri* (Allaker and Ian-Douglas, 2009).
	Chronic Periodontitis (CP)	The clinical influence of using probiotics as an adjunctive therapy of scaling and root planning (SRP) in the treatment of CP was investigated. Findings have supported the use of *L. reuteri* as an adjunct therapy with SRP in the treatment of CP at short terms (Martin-Cabezas *et al*., 2016).

Table 1.3 Continued

Health Area	Application	Function
	Reduction of high cholesterol	Growing, resting and dead cells of *L. plantarum* EM isolated from kimchi were investigated for their ability to remove high cholesterol. Removal of cholesterol was reported in all categories of *L. plantarum* EM investigated based on the high cholesterol-binding capacity of their cell wall fraction. *L. plantarum* was suggested to be a potential probiotic organism to reduce serum cholesterol regardless of its viability (Choi and Chang, 2015).
	Irritable bowel syndrome (IBS)	*B. infantis* 35624 is the only probiotic organism that has been reported to have the potential to significantly improve IBS symptoms (Gogineni *et al*., 2013). The efficacy of probiotics in IBS patients was investigated; distention, bloating and flatulence (DBF) were evaluated using an IBS severity scoring system to compare the effect of probiotics therapy in IBS patients to the placebo. The results showed that probiotic therapy was effective in improving DBF and a reduction in disease-associated complications in IBS patients was also reported (Didari *et al*., 2015).
	Reduction of lactose intolerance	Lactase deficient subjects have shown tolerance for lactose in yoghurt better than the same amount of lactose in milk. It has been suggested that the yoghurt is either supplying preformed lactase or bacteria that produce lactase in the small intestine. Experiments have shown *L. acidophilus* to improve lactose intolerance (Fuller, 1991).
	Allergies	27 breast-fed infants with atopic eczema were randomly grouped and weaned with either probiotic (*B. lactis* Bb12 or *L. rhamnosus*) supplemented extensively hydrolysed whey formula or without the probiotic supplement. The scoring atopic dermatitis score which measure the severity of eczema was significantly lower in the two probiotic groups compared to the control group after two months of therapy (Marteau *et al*., 2002)

1.5 Challenges of Maintaining Viability of Probiotics and Recent Approaches

Regarding the different definitions of probiotics, emphasis is being laid on the importance of "adequate" amount of bacteria administered and reaching the target site. This is because the benefits derived from consuming these bacteria are dependent on their survivability, ability to colonise and multiply in the host (Anal and Singh, 2007).

Most of the probiotic organisms applied in food and drink products do not survive for a long period and therefore this may compromise the adequate amount needed. The viability of probiotic bacteria is affected by several factors which include strain sensitivity to process factors (pH, fermentation temperature and oxygen), food matrix composition (water activity, pH, presence of natural antimicrobials, nutrient availability) and packaging and storage conditions. In addition, the GIT conditions (low pH of the stomach and exposure to bile salts in the small intestines) also contribute a reduction of viability of the probiotic bacteria (Figueroa-Gonzalez *et al*., 2011; Patrignani *et al*., 2017).

1.5.1 Viability during Freeze Drying

Freeze drying is a popular method of preserving microorganisms due to excellent long-term viability in most cases and the storage and distribution requirements are simple (Miyamoto-Shinoara *et al*., 2000). In addition, this technique offers lower transport and storage costs of the dried products and stable cultures in terms of viability and functional activities are obtained using this technique (Otero *et al*., 2007).

This method that is based on sublimation involves three phases namely: freezing, primary drying and secondary drying has been used for decades to manufacture probiotic powders (Meng *et al*., 2008, Martin *et al*., 2015).

Probiotic survival rates are typically higher using the freeze-drying technique, since the conditions are milder compared with other methods. However, the freezing and drying processes to prepare freeze dried bacteria are detrimental to cell structure and viability (Saarela *et al*., 2005; Meng *et al*., 2008). Osmotic shock and membrane injury resulting from intracellular ice formation and recrystallization are the major factors that contribute to loss of viability during freeze drying (Saarela *et al*., 2005). Another side effect associated with the freeze-drying technique is denaturation of sensitive proteins (Carvalho *et al*., 2004).

1.5.2 Viability during Storage

The viability of probiotic bacteria decreases due to different factors such as composition of the food, packaging material and storage environment (storage temperature, moisture content of powders, relative humidity, oxygen content, and exposure to light, among others) (Tripathi and Giri, 2014).

Foods containing probiotics aid the transport of probiotic bacteria to the target site. The physicochemical attributes of such foods (e.g. fat and protein content, sugars, pH; certain food additives such as sweeteners, salts, flavoring and coloring agents, lysozyme and nitrite) may have an effect on the functional attributes of probiotic strains and hence affect their performance and modify their functionality and efficacy (Vinederola *et al*., 2011, Tripathy and Giri, 2014).

In addition, it is important to note that most probiotic species are either anaerobic, strictly fermentative or saccharoclastic, hence exposure to molecular oxygen is detrimental to the growth and survival of some strains (de Vuyst, 2000). The effect of oxygen on probiotics can be due to toxicity of oxygen to some cells, production of toxic peroxides in certain cultures or production of free radicals as a result of oxidation of components such as fats. During storage

of probiotic products, the oxygen content in the package should be at a low level to avoid cell death and hence, loss of functionality of the product. Moisture content also affects the shelf-life stability of live bacteria. Water content left after drying affects the rate of loss of viability during storage as well as after drying (Tripathi and Giri, 2014).

Other factors that affect viability of probiotic organisms during storage include storage temperature, pH and titratable acidity and packaging (Tripathi and Giri, 2014).

1.5.3 Viability after Ingestion

For probiotics to exert their health benefits they must not only be able to survive processing and storage, they should also be able to survive transit through the acidic condition of the stomach, resist degradation by hydrolytic enzymes and bile salts in the small intestine before reaching their target site (Vasiljevic and Shah, 2008; Gandomi *et al*., 2016).

Probiotic organisms barely survive at pH below 4.4; however, this is strain specific as *L. acidophilus* is more resistant to low pH due to its high cytoplasmic buffering capacity while *Bifidobacterium spp.* are very susceptible to low pH. In the very high acidic environment present in the stomach, the growth of *Bifidobacterium spp.* is greatly hindered (Rius, *et al*., 1994). Different studies have reported loss of viability when probiotic organisms are exposed to simulated gastric juice. Su *et al*. (2011) reported a total loss in viability after 2 hours when *B. longum* BIOMA 5920 was exposed to simulated gastric juice, Gbassi *et al.* (2009) reported approximately ≈1 Log CFU/ml after 1 hour and total loss of viable cells was recorded after 90 minutes when *L. plantarum* strains were exposed to simulated gastric juice.

1.5.4 Maintaining Viability of Probiotic Organisms

Different techniques have been employed by researchers to improve the viability of probiotic organisms.

Cryoprotectants could be used during the freeze-drying process to reduce loss of viability of probiotic cells. Cryoprotectants are able to accumulate within the cells thereby causing a reduction in osmotic difference between internal and external environment (Martin *et al*., 2015). Capela, *et al*., (2006) reported an improvement in the viability of *L. casei* 1520 when Unipectin^{TM} RS 150 was used as a cryoprotectant. Some other cryoprotectants that have been used to maintain probiotic viability include sucrose, skimmed milk, trehalose, bacterial cellulose, dimethyl sulphoxide, polyvinylpyrrolidine and glycerol (Fijalkowski *et al*., 2016; Gisela *et al*., 2014; Jaganath *et al*., 2010).

Microencapsulation is one of the methods used for maintaining probiotic viability. It is a process that involves retaining of cells within an encapsulation membrane in order to reduce cell injury or cell loss such that appropriate release of cells in the gut takes place (Martin *et al*., 2015).

Microencapsulation provides an effective barrier that protects the cells from different stresses during the different stages of production, storage, low pH, high acidity, bile salts and also from degradation by elements of external environment (Tripathy and Giri, 2014). In addition to protection against adverse environmental factors, another aim of probiotic encapsulation is to enhance cell release in a viable and metabolic active state at the target site (Burgain *et al*., 2011).

The viability of encapsulated probiotic cells largely depends on the physico-chemical properties of the capsule, type and concentration of the coating material, particle size, bacterial strain and initial cell numbers. Some of the materials used for probiotic encapsulation include alginate, gellan gum and xanthan gum, cellulose acetate phthalate, chitosan, starch, K-carrageenan, gelatin and milk proteins. Encapsulation techniques include spray drying, extrusion, spray congealing, emulsification, extrusion, co-extrusion and spray coating (Anal and Singh, 2007; Burgain *et al*., 2011).

Spray drying is one of the encapsulation techniques which involves injecting liquids in atomised form into a hot drying medium at temperatures up to about 200°C. Exposure to very high temperatures can be detrimental to live probiotic cells, however, reports have shown a survival of ≥ 80% during spray drying of *L. paracasei* NFBC in reconstituted skimmed milk at outlet temperatures of 85°C - 90°C (Tripathy and Giri, 2014).

Large amount of material can be produced through spray drying which makes it economical; however, it is not often used because of records of high cell mortality (Anal and Singh, 2007).

The extrusion method of microencapsulation uses hydrocolloids as encapsulating materials and it is the most popular due to its simplicity, low cost and gentle formation that results in high viability (Martin *et al*., 2015). It involves projecting the solution containing probiotic cells through a nozzle at high pressure. Due to the slow formation of the microbeads, the extrusion method is difficult to use in scale-up production (Burgain *et al*., 2011).

The large size of microbial cells (about 1μm - 4μm) is one of the limiting factors for cell encapsulation. For capsules of size less than 0.1mm, loading sufficient cells is an issue, with larger capsules of >1mm, their application in food can have an impact upon food texture.

(Anal and Singh, 2007). Other limitations to the different microencapsulation techniques include irregular bead size, difficulties in mastering the techniques, damage from freezing, heat damage of bacteria and the need of specific equipment (Martin *et al*., 2015).

Addition of appropriate prebiotics is another alternative method of improving viability of probiotic cells in the food product and the subsequent delivery to the GIT. Prebiotics are defined as non-digestible substances that provide a beneficial physiological effect on the host by selectively stimulating the favorable growth or activity of a limited number of indigenous bacteria (Reid, 2008). Prebiotics such as inulin, oligofructose, and fructooligosaccharide (FOS) have been studied, and they have been reported to significantly increase faecal bifidobacteria at fairly low levels of consumption (5g - 8g per day) (Slavin, 2013). Improvement in viability of probiotic organisms was reported when Hi-maize, inulin or FOS (Raftilose P95) were added to yoghurt. FOS was the most effective of the prebiotics investigated and improvement of probiotic viability by 1.42 Log was recorded during four weeks of storage at 4°C (Capela *et al*., 2006).

Dark and milk chocolates have also been used as a protective carrier for oral delivery of probiotic bacteria *L. helveticus* CNCM I-1722 and *B. longum* CNCM I-3470 (Possemiers *et al*., 2010). Cells were embedded in dark and milk chocolates and passed through simulated gastric juice and simulated intestinal juice for 3.5 hours. It was reported that both types of chocolates provided better protection to the cells, although, milk chocolate provided a better protection to the cells compared with dark chocolates (Possemiers *et al*., 2010). The only limitations to using milk chocolates for probiotic delivery are lactose intolerance and diabetic patients.

Some reports have shown that probiotic bacteria, regardless of their viability, are able to carry out health benefits (de Vrese *et al*., 2006; Kumar *et al*., 2012; Reid *et al*., 2003) such as

reduction of duration and severity of common cold by immune stimulating effects of living or dead probiotic bacteria (de Vrese *et al*., 2006). Reid *et al*. (2003) reported 88% and 87% eradication of *H. pylori* in patients that received live *L. acidophilus* supplements and dead *L. acidophilus* supplements respectively compared with 72% eradication in antibiotic treated patients.

The mechanism of action especially with the dead *L. acidophilus* supplements is still unclear but it is assumed that the bacteria either inhibited the spread of *H. pylori* via competitive adhesion to glycolipid receptors or induction of host response that was detrimental to survival of *H. pylori* (Reid *et al*., 2003).

In the alleviation of lactose intolerance, the intervention of probiotics is assumed to take place in two levels which are hydrolysis of lactose in the milk product and in the small intestine or at the level of colonic fermentation. The actual amount of lactose in the dairy product such as yoghurt can be reduced by the hydrolytic capacity of some probiotic strains. The probiotic strains do not need to be alive but the membranes must be intact, this helps to protect β-galactosidase (it hydrolyses lactose) during gastric passage whilst for colonic fermentation, probiotic bacteria can be used to manipulate colonic fermentation. This mechanism will require the metabolism of substantial amount of probiotic bacteria in the gut (Vonk *et al*., 2012).

An expert consensus document (Hill *et al*., 2014) agreed that the development of metabolic by-products, dead microorganisms or other nonviable products may have potential effects, however, they do not fall under the probiotic construct (Hill *et al*., 2014), this makes probiotic viability and administration in the right dose important.

However, for some health benefits such as improving ulcerative colitis (UC) and Crohn's disease which are forms of irritable bowel disease (IBD), reduction of blood cholesterol and reduction of lactose intolerance, it is imperative to have a higher dose of probiotic bacteria for effectiveness.

It has been reported that there is a reduction of bifidobacteria and lactobacilli in the intestinal microbiota of IBD patients (Wang *et al*., 2014). Similar reports by Mylonaki *et al*. (2005) also showed a reduction in the number of bifidobacteria and an increase in *E. coli* counts in the rectal mucosa of UC patients compared with healthy individuals. Improving UC symptoms with probiotic treatment will require a higher dose of probiotic bacteria for active reinforcement of the epithelial barrier, inhibition of proinflammatory cytokine secretion as well as modulation of immune responses (Wang *et al*., 2014). Reoccurrence of ulcerative colitis has been reported to be managed by administration of bifid triple viable capsule to patients with UC (Cui *et al*., 2004).

Pagnini *et al*. (2010), gave a similar report that the promotion of gut health by the stimulation of epithelial innate immunity also requires a high dose of probiotics. They investigated the effect of low dose or a high dose of probiotic formulation (VSL #3, made up of eight different bacterial strains) in preventing the onset of Crohn's disease like ileitis in mice. With the low dose of VSL #3, they reported some improvements of villous deformation and thickening of the muscularis mucosa but severe inflammatory infiltrate of the lamina propria was still present with no significant difference from the control. Whilst with high dose of VSL #3, they recorded significant reductions in the severity of ileitis, villous deformation, active and chronic inflammations. Ileitis was completely prevented in 5 out of 11 mice treated. A near total protection of mucosal integrity and virtually no inflammatory cell infiltrates in the lamina propria were recorded in mice administered high dose VSL #3.

However, in mice with established ileitis, there was no significant difference between mice treated with high dose VSL #3 and the untreated mice. It was therefore concluded that high dose probiotic formulations such as VSL #3 are able to prevent the development of CD-like ileitis in SAMP mice by the stimulation of epithelial innate immunity (Pagnini *et al*., 2010).

One of the proposed mechanisms employed by probiotic bacteria in lowering blood cholesterol is by cholesterol assimilation (Oner *et al*., 2014), the requirement for a high dose of probiotic bacteria (10^{10} cells) in lowering blood cholesterol via cholesterol assimilation was reported by Tomaro-Duchesneau *et al*. (2014). Their study investigated the potential ability of selected *Lactobacillus* species to assimilate cholesterol under simulated gastrointestinal conditions. It was reported that all the investigated strains (excluding *L. rhamnosus* ATCC53103 GG) assimilated cholesterol significantly. *L. reuteri* NCIMB 701089 showed the most assimilation with 2254.70±63.33mg of cholesterol per 10^{10} cells (Tomaro-Duchesneau *et al*., 2014). This reports clearly demonstrates a requirement for a high dose of probiotics.

Another similar report by Miremadi *et al*., (2014) and Choi and Chang (2015) showed that growing, resting and dead cells are able to reduce high cholesterol levels significantly. However, Miremadi *et al* (2014) reported that cholesterol assimilation by growing cells was significantly higher than in resting and dead cells. This was also supported by the reports of Kumar *et al*. (2012) that growing cells removed more cholesterol than heat killed cells.

Probiotic products have been marketed in some yogurts and some as tablets. It is important to note that individuals who are lactose intolerant will be sceptical about taking yoghurt containing probiotics due to their health situation and also not all individuals are comfortable with taking tablets or supplements.

These health benefits discussed and the reasons highlighted are potential targets for microencapsulation since a high dose of live bacteria will be required to carry out some of the health benefits associated with consumption of probiotics. Microencapsulation techniques such as freeze drying will not only be a means of transporting probiotic bacteria to the target site, it will also provide long term viability.

In recent years, research has focused upon developing ways of tackling loss of probiotic viability during the different stages of production and during ingestion, since probiotic organisms can only exert their health benefits only if they get to the target site in the right amount. It is a challenge for microbiologists and food technologists to monitor the functionality of probiotic organisms during all stages of production to the addition to food products and eventually during the consumption stage (Vinderola, 2011).

2.0 INTRODUCTION TO POLY-γ- GLUTAMIC ACID (γ-PGA)

2.1 Background

Polyglutamic acid (PGA) has been defined as a biodegradable non-immunogenic and unusual anionic homopolyamide that is made up of D and or L glutamic acid units. It has no fixed molecular weight and it is only made up of glutamic acid residues (Shih and Van, 2001).

There are two isoforms of PGA (α-PGA and γ-PGA) depending on the attachment of amino group to the carboxyl group (Figure 2.1). γ-PGA is usually synthesised by bacteria, in contrast, α-PGA is chemically synthesised by nucleophile initiated polymerization of the γ-protected N-carboxyanhydride of L-glutamic acid. Microbial production of α-PGA is difficult; hence it can only be produced through recombinant technology (Buescher and Margaritis, 2007).

Poly-gamma glutamic acid was first discovered by Ivanovics and his co-workers (Shih and Van, 2001). It was discovered as a capsule of *Bacillus anthracis,* which upon aging and autolysis of the cells (or autoclaving) was released into the medium. γ-PGA is a biodegradable, edible, water-soluble biopolymer that is non-toxic to humans and to the environment (Feng *et al*., 2014).

It can be classed as a pseudopolyamino acid that only consists of repeating units of glutamic acid connected by amide linkages (Figure 2.1), and can exist either in its free acid or its salt form. The salt forms can be Na^{+}, Mg^{++}, NH_4^{+} or Ca^{++} depending on the growth medium (Kedia *et al*., 2010). Figure 2.2 shows the sodium and the acid form of γ-PGA.

(a) **(b)**

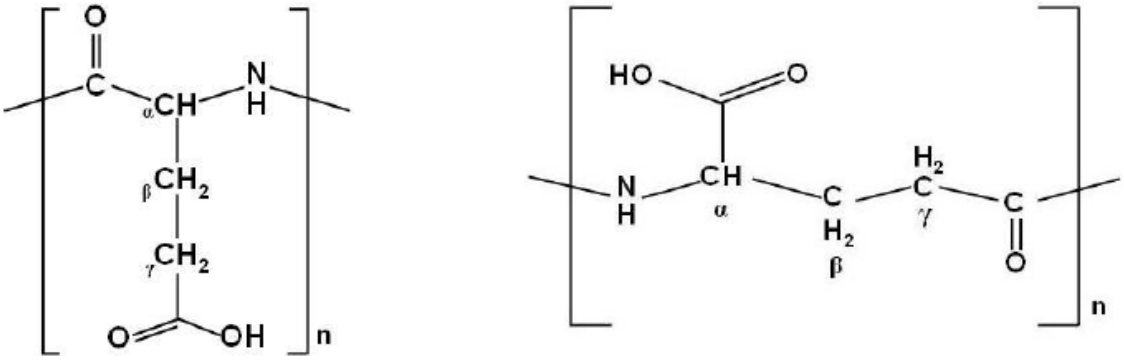


Figure 2.1: (a) Structure of α-PGA where glutamic acid residues are connected via amide linkages between α-amino and α-carboxylic acid groups (b) Structure of poly-gamma-glutamic acid (γ-PGA) glutamic acid residues are connected through amide linkages between α-amino and γ-carboxylic acid groups. (n~10,000) (Ogunleye *et al*., 2015).

γ-PGA is frequently found in the mucilage of *natto*, a Japanese traditional fermented food (Ashiuchi, 2013). γ-PGA is secreted freely into growth medium of *Bacillus subtilis* as a fermentation product; several *Bacillus* species have demonstrated γ-PGA production outside the cells. It is also found in *Natrococcus occultus* and the nematode hydra, γ-linked glutamic acid polymers have been identified where it is found attached to folic acid and it is found as an appendage to the structural protein tubulin (Shih and Van, 2001).

2.2 Properties of γ-PGA

γ-PGA is a naturally occurring, anionic homo-polyamide that is made of only D-, only L- or both enantiomers of glutamic acid linked together by amide linkages between the α- amino and γ- carboxylic groups as shown in figure 2.2 (Shih and Van, 2001). γ-PGA is an optically active polymer that possesses a chiral centre in every glutamate residue (Sung *et al*., 2005).

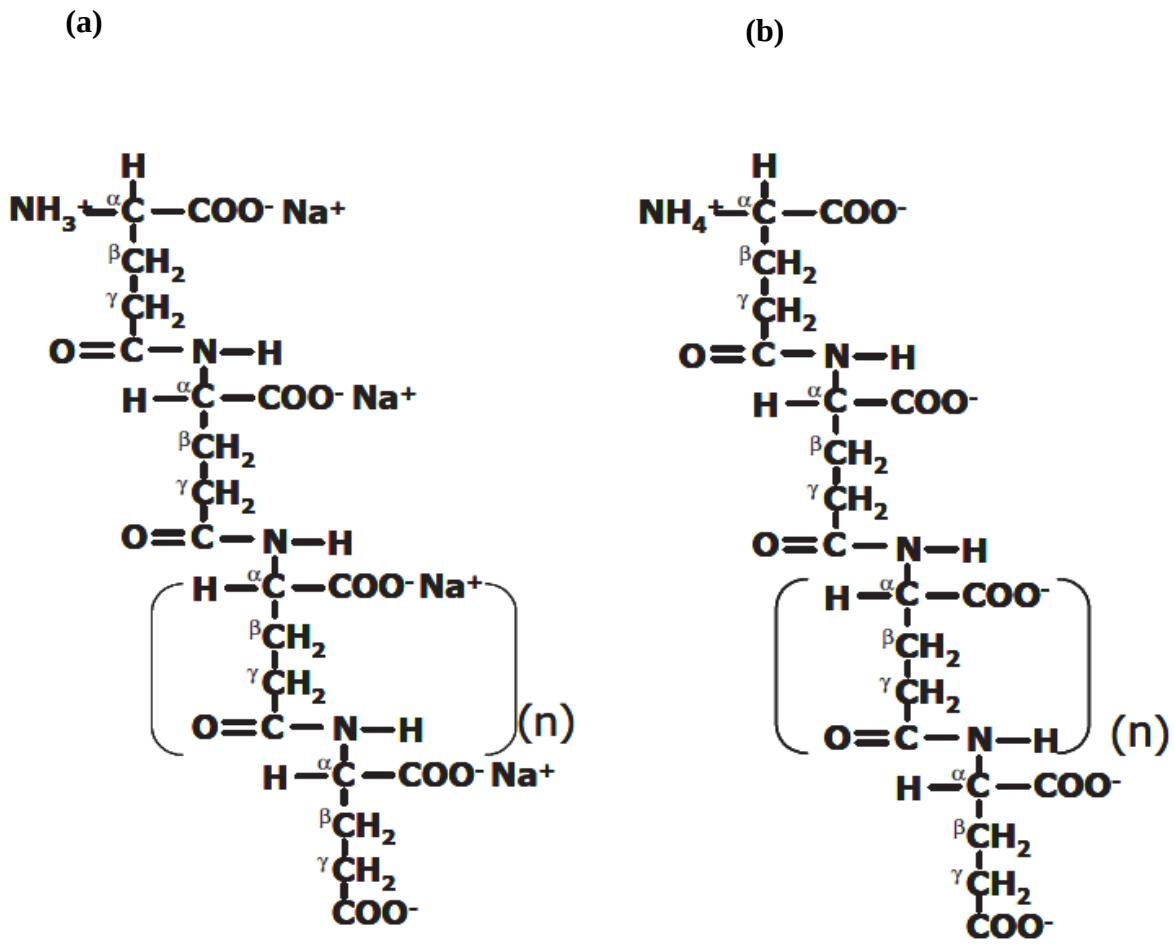


Figure 2.2: (a) Sodium salt form of γ-PGA and (b) acid form of γ-PGA. (n~1000) (Sung *et al.*, 2005).

γ-PGA is freely soluble in water and because of its α-carboxylate side chains, it is able to bind a wide range of metals ions with high affinity (King *et al*., 1997). It is biodegradable (Buescher and Margaritis, 2007), edible and non-toxic to humans and to the environment (Bajaj and Singhal 2011). It also contains high anionic charge number which allows it to form a polyelectrolyte complex hydrogel with chitosan in an appropriate pH range (Tsao *et al*., 2011).

γ-PGA is resistant to proteases which cleave only α-amino bonds, but it is broken to monomers of glutamic acid in mild acidic conditions (Li, 2002). γ-PGA is highly soluble;

sequesters water molecules and it can be of high molecular weight (up to about 10,000 kDA). Electrophoresis on paper can be used to separate γ-PGA from other neutral components (Zeng *et al.*, 2016; Candela and Fouet, 2006). Another property of γ-PGA which makes it different from proteins is its ability to be stained with methylene blue and not Coomasie blue (Kedia *et al*., 2010).

It has been reported that γ-PGA is able to adopt several structures. Studies of purified γ-PGA from *B. licheniformis* showed its conformation as flexible depending on the γ-PGA concentration and pH of the solution. γ-PGA adopts a conformation solely based on α-helices at pH below pH 7.0 and low concentration and a β-sheet based conformation at higher pH. The negative charges of PGA are efficiently revealed by the β-sheet conformation (Candela and Fouet, 2006).

Depending on the producing organism, the molecular sizes of γ-PGA filaments appear to differ. This variation is probably due to varying γ-PGA degradation or variation in the purification and analysis methods used. High performance liquid chromatography (HPLC) overestimates the size of γ-PGA, this is probably due to a difference in the 3D structure between γ-PGA and poly-α-glutamic acid (Tanaka *et al*., 1997).

Since γ-PGA may contain either the D- glutamate, L- glutamate or both enantiomers, γ-PGA filaments may also be poly-γ-L-glutamate (PLGA) filaments, poly-γ-D-glutamate (PDGA) filaments or poly-γ-L-D-glutamate (PLDGA) filaments. PLGA and PDGA are soluble in ethanol, if however, both are mixed in equimolar proportion; they are able to precipitate in ethanol. This was used to show that γ-PGA from *B. licheniformis* is made up of PDGA and PLGA filaments. However, studies using L-γ-glutamyl hydrolase digestion have shown that γ-PGA from *B. subtilis* consists of PLGA and PLDGA filaments (Candela and Fouet, 2006).

2.3 γ-PGA Producing Organisms

γ-PGA producing organisms are divided into two categories: L-glutamic acid dependent and L-glutamic independent. Most of the known γ-PGA producers belong to the first category (Cao *et al*., 2010) while a few strains have been identified to belong to the second category.

γ-PGA is mainly produced by bacteria belonging to the *Bacillus* species (Kocianova *et al*., 2005). Other organisms reported to produce γ-PGA include: two halophilic eubacteria (*Sporosarcina halophilia* and *Placococcus halophila*) and *Natrialba aegyptiaca* (Bajaj and Singhal 2011; Feng *et al*., 2007).

Non- anchored γ-PGA are known to be produced by several organisms (bacteria, one archaea and one eukaryote) (Candela and Fouet, 2006). All bacteria that have been identified to produce γ-PGA are Gram-positive, belonging to the bacillales order and to bacilli *c*lass, hence are all phylogenetically closely related (Candela and Fouet, 2006). *Fusobacterium nucleatum*, has been reported to be the first Gram- negative bacterium to demonstrate γ-PGA production (Candela *et al*., 2009).

Organisms reported to produce γ-PGA, type, role and conformation of produced γ-PGA, are summarised in table 2.1.

Table 2.1: γ-PGA producing organisms (Adapted from Candela and Fouet, 2006; Candela and Fouet, 2009; Kimura *et al*., 2004; Weber, 1990; Buescher and Margaritis, 2007.

Organism	Function of γ-PGA Produced	Anchored or Released	Conformation	Conformation Filament
Planococcus halophila	Reduction of high local salt concentration which enhances survival in hostile environment	Released	D	D
Sporocarcina halophila	Reduction of high local salt concentration which enhances survival in hostile environment	Released	D	D
Natrialba asiatica	Reduction of high local salt concentration which enhances survival in hostile environment	Released	L	L
B. subtilis	Can be a source of glutamic acid	Released	D and L	L and D+L
B. anthracis	Possesses a capsule that is made of γ-PGA. This capsule acts as protective barrier against antibodies and prevents it against phage infection	Anchored	D	D
S. epidermidis	Protects the cocci against antimicrobial peptides. Acts as a virulence factor as it allows the bacteria to escape phagocytosis	Anchored	D and L	Not determined
B. mesentericus	Toxic metal ions sequestration. This increases resistance to adverse environmental conditions	Released	D	D
B. licheniformis	Toxic metal ions sequestration. This increases resistance to adverse environmental conditions	Released	D and L	D and L
B. pumilus	Toxic metal ions sequestration. This increases resistance to adverse environmental conditions	Released	D and L	Not determined
Fusobacterium nucleatum	The only known gram negative bacteria that produces γ- PGA	Not determined	Not determined	Not determined
Hydra (Cnidaria and Hydrozoa)	Triggers explosive organelles that are used for locomotion, defence and prey capture	Released	Not determined	Not determined
Neurons of Mice	Regulation of microtubule dynamics through modification of the tubulin-associated proteins and Ca^{+2} interactions	Not determined	Not determined	Not determined

2.4 Synthesis of γ-PGA

2.4.1 Genes Associated with γ-PGA Synthesis

In order to understand the biosynthesis and secretion of γ-PGA, it is important to identify the genes and determine the location of enzymes related to γ-PGA synthesis (Feng *et al*., 2007).

Depending on whether the γ-PGA synthesised is released or retained, the name for the genes involved is either of two types. If the γ-PGA is associated with the bacterial surface and forms a capsule, then the genes are named "*cap*" (capsule); however, if the γ-PGA associated is released, the genes are named "*pgs*" (**p**oly**g**lutamate **s**ynthase) (Candela and Fouet, 2006) (Figure 2.3).

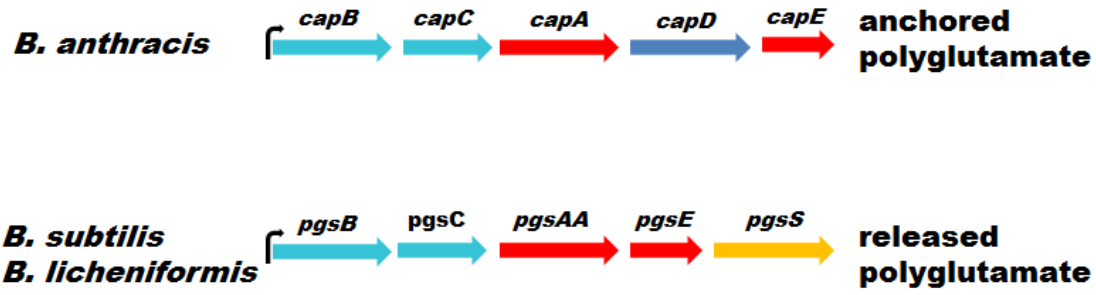


Figure 2.3: Required genetic elements for γ-PGA synthesis in *B. anthracis, B. subtilis* and *B. licheniformis* (Adapted from Candela and Fouet, 2006)

In *B. anthracis*, the genes related to γ-PGA synthesis lie on a large plasmid DNA (pXO2). *CapB, capC, capA* and *capE* are four genes necessary for γ-PGA synthesis. In addition, these four genes are enough for synthesis of γ-PGA in a plasmid-less strain of *B. anthracis* (Shih and Van, 2001; Candela and Fouet, 2006).

It was reported that *B. subtilis* TAM-4 has no plasmid and the genes coding for γ-PGA synthesis lie on the genomic DNA. In *B. subtilis* (natto), the genes related to γ-PGA synthesis

have been described to be on plasmids found on the organism, it was however reported that the plasmids did not encode any gene required for synthesis of γ-PGA. It was then suggested that genes involved in the production of γ-PGA are probably present in the genomic DNA of *B. subtilis* (natto) (Shih and Van, 2001).

The enzymatic complex PgsBCA (polyglutamate synthetase complex) is responsible for the polymerization and transport of γ-PGA through the cellular membrane (da Silver *et al*., 2013). This pgs locus is enough for synthesis of γ-PGA; however, the presence of an additional small open reading frame (ORF) was revealed upon analysis of the cloned DNA. This ORF was named *pgsE*, by analogy to *capE* (Candela and Fouet, 2006).

The roles of *pgsB*, *pgsC* and *pgsA* have been studied. It has been reported that *pgsA* may function as a γ-PGA transporter, this is likely important for the extension of γ-PGA chain and secretion of γ-PGA. *PgsB* is thought to be the main catalytic component with a primary structure that looks like water soluble Mg/ATP dependent amide ligases that catalyse the addition of short γ-L-glutamyl chain to a folic acid moiety. *PgsC* is likely a membrane-embedded component of the enzyme and a structural resemblance to the N-acetyltransferase domain of N-acetylglutamic acid synthetase has been determined. The role of *pgsE* remains relatively unknown, it has been proposed that it might be functionally identical to a membrane-associated *capE*, an essential component of a plasmid borne PGA synthetic system in *B. anthracis*. A novel function of *pgsE* was recently discovered and it may be particularly significant in some plasmid-borne PGA synthetic systems (Ashiuchi *et al*., 2010).

The membrane locations predicted for these proteins are shown in figure 2.4. The orientation suggests that γ- PGA is synthesised in the cytoplasm and transported to the outer surface, it also suggests that γ-PGA polymerization and transport are the two functions required for γ-PGA synthesis (Candela and Fouet, 2006).

In capsular bacterial strains, such as *B. anthracis* and *S. epidermidis* (figure 2.4A) γ-PGA is anchored covalently to the peptidoglycan via *capD* which is localized in the membrane or peptidoglycan. While in released γ-PGA bacterial strains such as *B. licheniformis* and *B. subtilis,* (figure 2.4B) γ-PGA is found in the extracellular medium. *PgsS* has not been definitively shown to be present at the bacilli cell's surface hence the (?). This probably facilitates the release of γ-PGA (Candela and Fouet, 2006).

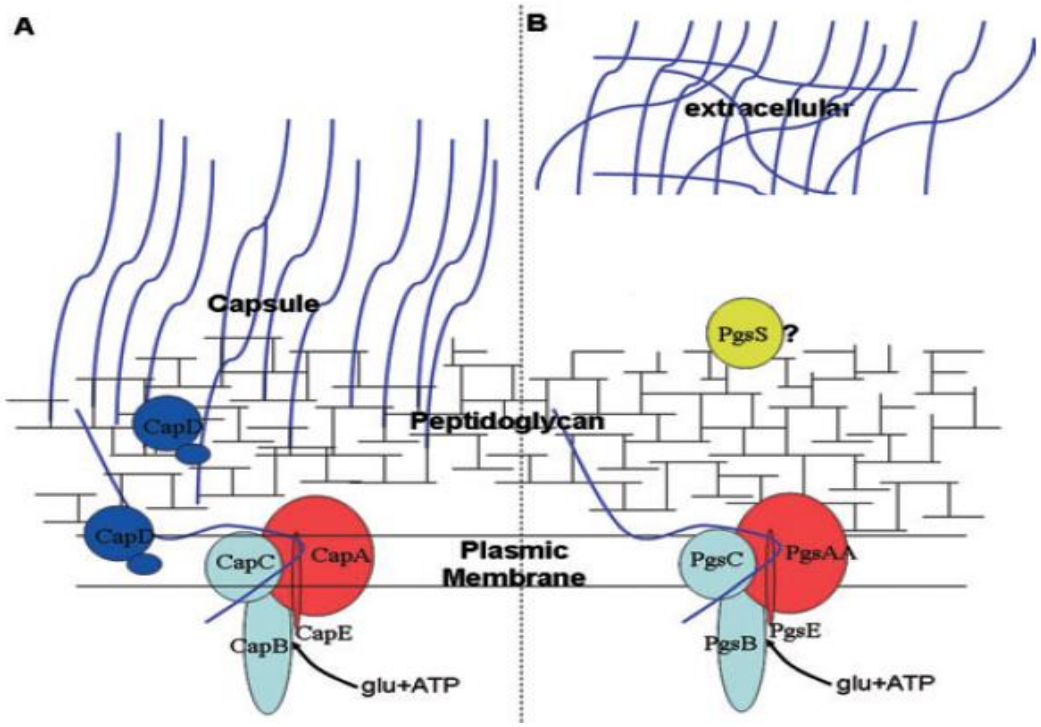


Figure 2.4: Schematic representation of γ-PGA synthesis membrane- anchored complex showing proteins according to their predicted orientation in the membrane. (A) γ-PGA covalently anchored to the peptidoglycan via *capD*, localized in the membrane or peptidoglycan, in *B. anthracis* and probably in *S. epidermidis*. (B) γ-PGA is found in the extracellular medium in *B. subtilis* and *B. licheniformis*. (adapted from Candela and Fouet, 2006).

2.4.2 Mechanism of γ-PGA Synthesis

γ-PGA is an extracellular product and the process of its synthesis is ribosome independent but enzyme catalysed (Feng *et al*., 2007). One of the major challenges in the industrial use of γ-PGA is its cost of production. The cost of production is about one hundred-fold higher than the currently used conventional materials γ-PGA is meant to replace (Sung *et* al., 2005). Overcoming this challenge would require a mass γ-PGA production system through molecular biology techniques, this will require an in-depth knowledge of γ-PGA producers, genes and enzymes involved in γ-PGA synthesis (Sung *et al*., 2005; Ogunleye *et al*., 2015).

A biosynthetic pathway has been proposed for the synthesis of γ-PGA (figure 2.5). L-glutamic acid units that make up γ-PGA are derived either exogenously or endogenously via the glutamic acid biosynthetic pathway using α-ketoglutaric acid as a direct precursor. The conversion of a carbon source via acetyl-CoA and tricarboxylic acid (TCA) cycle intermediates is required for the endogenous production of L-glutamic acid with α-ketoglutaric acid from the TCA cycle as a direct precursor of glutamic acid synthesis. Exogenous L-glutamic acid can be converted to L-glutamine with the help of the enzyme glutamine synthase. L-Glutamine is a precursor of γ-PGA as well (Luo *et al*., 2016).

The conversion of α-ketoglutaric acid is assumed to take place in two ways:

(1) When glutamine is absent, the glutamate dehydrogenase pathway is used in which L-glutamate is synthesised from α-ketoglutaric acid and ammonium sulphate, this reaction is catalysed by glutamate dehydrogenase.

(2) When L-glutamine is present another pathway involving glutamine synthetase and glutamine is used. 2-oxoglutarate aminotransferase is used. L-glutamine is formed

from α-ketoglutaric acid and L-glutamine. This is catalysed by 2-oxoglutarate aminotransferase (Shih and Van, 2001).

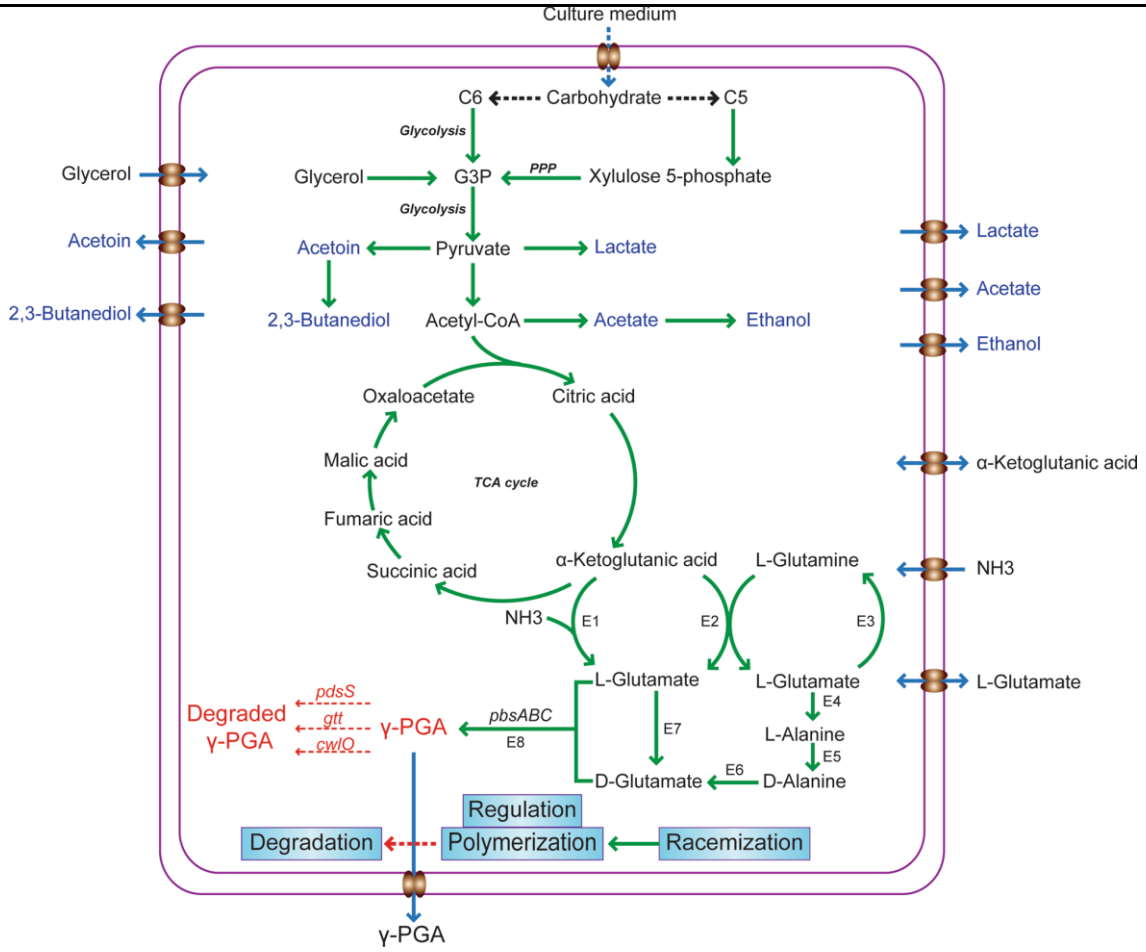


Figure 2.5: Biosynthetic pathway for microbial synthesis of γ-PGA. PPP: pentose phosphate pathway, G3P: glyceraldehyde 3-phosphate, E1: glutamate dehydrogenase (GD), E2: glutamate 2-oxoglutarate aminotransferase, E3: glutamine synthetase (GS), E4: L-glutamic acid: pyruvate aminotransferase, E5: alanine racemase, E6: D-glutamic acid: pyruvate aminotransferase, E7: direction conversion, E8: PGA synthetase (Luo *et al*., 2016).

Substrates in the culture medium include a variety of biomass materials, cane molasses, agro-industrial wastes that can be degraded into C6 and C5 compound. This enters the main carbon metabolism via glycolysis and pentose phosphate pathway (figure 2.5). Glycerol and metabolic intermediates of citrate cycle were also used as candidate substrates. The main by-

products were acetoin and 2, 3-butanediol; other by-products with little production were lactate, ethanol, and acetate (Luo, *et al*., 2016)

The mechanism of γ-PGA production can be seen to have four important steps (γ-PGA racemisation, γ-PGA polymerization, γ-PGA degradation and γ-PGA regulation) which will be explained in the sections below.

2.4.2.1 Racemization

γ-PGA is synthesised from either D- or L-glutamic acid or from both enantiomers. Therefore, a racemization reaction is required to convert L-glutamic acid (exogenous or endogenous) into D-glutamic acid to incorporate D-glutamic acid into the growing chain of L-glutamic acid unit (Luo *et al*., 2016).

Three possible mechanisms were previously proposed (Buescher and Magaritis, 2007) for the racemization of L-glutamic acid:

i. An indirect one by amino transferase
ii. A direct one by glutamic acid racemase (Glr or RacE)
iii. A direct one by glutamic acid racemase (Yrpc)

Studies have suggested that Glr/RacE is only responsible for the provision of the glutamic acid monomers (Buescher and Magaritis, 2007). The indirect route is unlikely to be taken *in vivo* because a decrease in activity of aminotransferase occurred during the production of γ-PGA in *B. subtilis* C1 while an increase in Glr activity was reported. Glr is a cytosolic enzyme with high selectivity for glutamic acid with preference for L- glutamic acid (Buescher and Magaritis, 2007).

Cultivation in the absence of Mn^{2+} yields γ-PGA of 90% L- glutamic acid content; this suggests that activity of the enzyme does not appear to be influenced directly by Mn^{2+}.

However, the expression of the Glr gene has been reported to be Mn^{2+} dependent (Buescher and Magaritis, 2007). Yrpc was found to be active when cells were grown on minimal medium (Ogunleye *et al*., 2015).

2.4.2.2 Polymerization

Polyglutamate synthase (pgs) (Figures 2.3 and 2.4) is encoded by four genes (*pgs*B, C, A, and E), their homologs in *Bacillus* species are *yws*C, *ywt*AB, and *cap*BCA. PgsBCA was recently identified as the sole machinery responsible for γ-PGA polymerization at the active site of the synthase complex (PgsBCA) in an ATP-dependent reaction (Luo *et al*., 2016).

PgsBCA cannot be isolated in an active state due to its high instability and hydrophobic nature but it has been described in detail. γ-PGA is formed in the membrane-bound form from glutamic acid and ATP, ATP cleaves to ADP as shown in figure 2.6 and they supply the driving force for the reaction (Buescher and Magaritis, 2007).

The phosphoryl group of ATP is initially transferred to a terminal carboxyl group of elongated γ-PGA through substrate-dependent ATP hydrolysis. Then, a nucleophilic attack of an amino group of glutamic acid on the phosphorylated carboxyl group results in the formation of an amide linkage (Sung *et al*., 2005).

This reaction continues to polymerize γ-PGA at the active site of the synthase complex (PgsBCA). *PgsB* and *pgsC* together form most parts of the complex's catalytic site, whereas, the elongated chain seems to be removed from the active site by *pgsA* so that the next monomer can be added and may also be involved in transporting γ-PGA (Ashiuchi et al., 2001; Ogunleye *et al*., 2015).

The activity of PgsBCA was found to be dependent on Mg^{2+}. Less compact cell membranes with shorter phospholipids can aid transportation of γ-PGA outside the cell (Buescher and Margaritis, 2007).

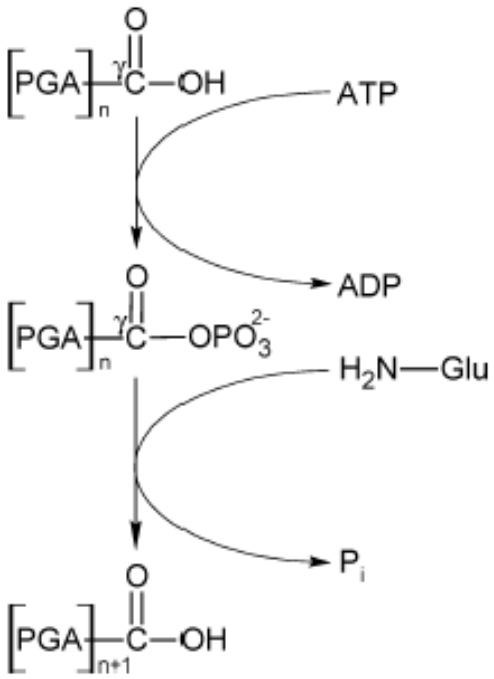


Figure 2.6: Proposed mechanism for the polymerization reaction in *B. subtilis* (Adapted from Buescher and Margaritis, 2007)

2.4.2.3 Regulation of γ-PGA Biosynthesis

A two-way signal transduction system is responsible for the regulation of γ-PGA synthesis; they are the ComP-ComA regulator, and the two-part DegS-DegU, DegQ, and SwrA system (Luo *et al*., 2016).

This involves the phosphorylation of the histidine residue of kinase molecule once signal is received. The phosphoryl group is then transferred to an aspartate residue of a cognate response regulator which usually acts as a transcription factor (Najar and Das, 2015).

DegSU, DegQ, ComPA appear to have transcriptional regulatory effects in response to quorum sensing, phase variation signals and osmolarity whilst that of SwrA appears to be post transcriptional (Stanley and Lazazzera, 2005).

Upon examination of the relationship between SwrA and DegU, it was revealed that the presence of SwrA and phosphorylated DegU (DegU-P) is important for full activation of the pgs operon and hence, γ-PGA production (Osera, *et al*., 2009). A high level of DegU-P has been reported to directly activate pgs expression instead of SwrA and high levels of DegQ; however, SwrA was discovered to be necessary for γ-PGA production under some experimental conditions (Ogunleye *et al*., 2015).

The role of DegQ in γ-PGA synthesis was identified by knocking out the DegQ gene in *B. subtilis* (*natto*) and replaced with suppressor mutants that can produce γ-PGA in the absence of degQ (Do, *et al*., 2011). This alteration of *deg*Q prevents the synthesis of γ-PGA and effectively down regulates the production of degradation enzymes. (Do *et* al., 2011).

2.4.2.4 Degradation of γ-PGA

Biodegradability is one of the special properties of γ-PGA. All strains producing γ-PGA are able to metabolize γ-PGA as carbon and nitrogen sources (Feng *et al*., 2007). The *pdgS* gene located downstream of the *pgs* operon has been identified to code for the γ-PGA degradation enzyme (Feng *et* al., 2014). Endo-γ-glutamyl-peptidase and exo-γ-glutamyl-peptidase are the two enzymes that can degrade γ-PGA in γ-PGA producing bacilli and they catalyse the transfer of the γ-glutamyl moiety of a γ-glutamyl compound to various amino acids, peptide receptors and water (Buescher and Magaritis, 2007; Feng *et al*., 2014 and Luo *et al*., 2016).

Endo-γ-glutamyl peptidase can be secreted into the medium by *B. subtilis* and *B. licheniformis*, where it is able to cleave high molecular weight γ-PGA into fragments of 1000

Da to 20 kDa, which decreases dispersity as a function of depolymerisation time while exo-γ-glutamyl peptidase is an important enzyme in the metabolism of glutathione. It catalyses the formation of γ-glutamic acid di- and tripeptides *in vitro*, but appears not to be involved in γ-PGA synthesis *in vivo* (Luo *et al*., 2016).

Chemically, γ-PGA can be degraded by prolonged exposure to extremely high pH and high temperature, physically; it can be degraded by ultrasonic irradiation or enzymatically. Although, aqueous solution of γ-PGA is stable at room temperature and in the presence of ordinary proteases but the amide will be broken when there is an increase in temperature up to about 60°C (Buescher and Magaritis, 2007; Feng *et al*., 2007).

An alternative to enzymatic hydrolysis is ultrasonic irradiation, when γ-PGA was exposed to 20,000 Hz for 2 hours, it was reported that this process cleaved γ-PGA of 2100 kDA into 60-90 kDA fragments. The fragments portrayed a narrowed polydispersity compared to the high molecular weight γ-PGA starting material (Buescher and Magaritis, 2007).

In the fermentation broth of *B. licheniformis* 9945A, a depolymerase activity was expressed; this appeared to be associated with the cell surface (Opperman *et al*., 1998), and the depolymerase has been identified to physically bind to the γ-PGA around the cell (Feng *et al*., 2007). *B. subtilis* IFO 3335 has also been reported to be able to degrade γ-PGA with the depolymerase being excreted into the medium (Opperman *et al*., 1998).

γ-PGA degradation by other natural organisms has also been investigated. 10ml of 0.5% (w/v) γ-PGA in mineral medium was inoculated with 100µl sewage sludge and incubated at 30°C for 3 days. 21 bacterial strains that make use of γ-PGA as carbon source were isolated and 12 of these strains have been clearly identified (Opperman *et al*., 1998).

2.5 γ-PGA Production by Microbial Fermentation

Different *Bacillus* species have been investigated for the production of γ-PGA. Based on the required nutrients, γ-PGA producing organisms have been classified into two groups: glutamic acid dependent bacteria and glutamic acid independent bacteria (Cao *et al*., 2011).

γ-PGA producing organisms that are glutamic acid dependent include: *B. anthracis*, *B. licheniformis* ATCC 9945A, *B. subtilis* IFO 3335 and *B. subtilis* F-2-01. γ-PGA producing organisms that are glutamic acid independent include: *B. subtilis* C1, *B. subtilis 5E*, *B. subtilis* TAM-4, *B. amyloliquefaciens* LL3 and *B. licheniformis* A35 (Shih *et al*., 2002; Cao *et al*., 2011; Cao *et al*., 2013).

B. subtilis 5E is able to produce γ-PGA using L- proline as the sole carbon source and nitrogen source in medium (Shih and Van, 2001). *B. licheniformis* A35 is able to produce γ-PGA from glucose and ammonium chloride under denitrifying conditions and *B. subtilis* TAM-4 is able to produce γ-PGA in a culture medium that contains salt and sugar as nitrogen and carbon sources (Shih and Van, 2001).

Apart from glutamic acid, other factors such as carbon and nitrogen sources, ionic strength, agitation, aeration and pH of the medium affect the quality and production yield of γ-PGA (Bajaj and Singhal, 2011).

γ-PGA production has been studied extensively in glutamic acid dependent organisms, table 2.2 summarizes the nutrient requirements, cultivation conditions, yield and molecular weight of γ-PGA produced from the different γ-PGA producing organisms.

Table 2.2: Report of the different strains of γ-PGA producing bacteria, showing the nutrients involved, conditions of cultivation, productivity and molecular weight of the produced strains of γ-PGA (Adapted from Shih and Van, 2001).

Strain	Nutrients	Cultivation Conditions	Yield (g/l)	Molecular Weight
B. licheniformis ATCC 9945	Glutamic acid, glycerol, citric acid, NH_4Cl	30°C, 4 days	17-23	$1.4x10^5$-$9.8x10^5$
B. subtilis IFO3335	Glutamic acid, citric acid	37°C, 2 days	10-20	$1.0x10^5$-$2.0x10^6$
B. subtilis TAM-4	Fructose, NH_4Cl	30°C, 4 days	20	$6.0x10^5$-$1.6x10^6$
B. licheniformis A35	Glucose, NH_4Cl	30°C, 3-5 days	8-12	3.0~$5.0x10^5$
B. licheniformis A35	Glutamic acid, glucose	30°C, 2-3 days	50	$1.20x10^6$
B. subtilis natto	Maltose, soy sauce, sodium glutamate	40°C, 3-4 days	35	Not determined

2.5.1 γ-PGA Production by Glutamic Acid Dependent Organisms

2.5.1.1 Production of γ-PGA *B. licheniformis* ATCC 9945A

B. licheniformis ATCC 9945A (NCIM 2324) is a common strain that has been used for the production of γ-PGA. Factors such as glutamic acid, citric acid, inorganic salts, glycerol and size of inoculum have been reported to affect the production of γ-PGA by *B. licheniformis* ATCC9945A in shake flasks and static cultures (Ogunleye *et al*., 2015; Shih and Van, 2001).

γ-PGA yield in excess of 15g/l was reported when the organism was grown in shake flasks in medium C (medium C contains: 20g/l L-glutamic acid, 12g/l citric acid, 80g/l glycerol, 7g/l NH_4Cl, 0.5g/l $MgSO_4.7H_2O$, 0.04g/l $FeCl_3.6H_2O$ and 0.5g/l K_2HPO_4), tap water and pH adjusted to 7.4 with NaOH. Investigations revealed that maximum polymer yield and

maximum growth were obtained when a specific lot of $FeCl_3$ and tap water were used during medium preparation, further studies then revealed that a significant amount of Ca^{2+} was present in the tap water and that the $FeCl_3$ was contaminated with Mn^{2+} (Shih and Van, 2001).

Further investigations were carried out on the role and optimal concentrations of Mn^{+2} and Ca^{2+} in medium C for γ-PGA synthesis by *B. licheniformis* ATCC 9945A. Results showed that only 1.5 $x10^{-7}$M of Mn^{2+} was required for maximum cell growth, increasing the concentration had an effect on cell viability and thus increased the γ-PGA yield. Increasing the concentration of Mn^{2+} to 6.15 $x10^{-4}$M resulted in maximum yields of γ-PGA. Addition of 1.02 $x10^{-3}$M of Ca^{2+} in the presence of 1.5 $x10^{-7}$M of Mn^{+2}M resulted in maximum yields of γ-PGA. Based on these results, medium C was modified into medium E (see table 4.3 for the composition of medium E) and this has become the choice medium for γ-PGA production (Shih and Van, 2001).

In another report, production of γ-PGA was optimised with *B. licheniformis* NCIM 2324 via the 'one factor at a time' method. This method was used to examine the effect of carbon sources, nitrogen sources and pH on γ-PGA production. The response surface method was then used to devise the optimum nutrient concentrations. γ-PGA yield of 26.12g/l with a molecular weight of $2.1x10^5$ Da was obtained with the devised medium compared with a yield of 5.27 with the basal medium (Bajaj *et al*., 2009).

2.5.2 γ-PGA Production by Glutamic Acid Independent Organisms

Bacteria that do not require glutamic acid to produce γ-PGA are of importance because of the reduced cost of production and simplified process in industrial fermentor production systems. (Cao *et al*., 2011).

2.5.2.1 Production of γ-PGA by *Bacillus subtilis* TAM-4

B. subtilis TAM-4 that was isolated from the soil was reported by Ito and co-workers (1996) to produce a large amount of γ-PGA if grown aerobically in a culture medium that contains sugar and ammonium salt as sugar and nitrogen sources respectively. In contrast to other γ-PGA producing strains, *B. subtilis* TAM-4 does not require biotin for its growth and does not have strain degeneration challenges. This strain has the long ability to produce γ-PGA; it is maintained by semi-annual sub-culturing on trypticase soy broth (TSB) slants at room temperature. *B. subtilis* TAM-4 can use several sugars and organic/inorganic nitrogen sources for production of γ-PGA. Ammonium chloride and fructose are the most favourable nitrogen and carbon sources respectively (Shih and Van, 2001).

A maximum γ-PGA yield of 22.1g/l was obtained when *B. subtilis* TAM-4 was grown in medium M (1.8% ammonium chloride, 7.5% glucose, 0.15% K_2HPO_4, 0.035% $MgSO_4.7H_2O$, 0.005% $MnSO_4 \cdot 5H_2O$, and 3.0% $CaCO_3$ (pH 7.2)), at 30°C for 96 hours and at 150 rpm with shaking. This high yield is distinct especially with the absence of glutamic acid in the medium. Upon testing of the produced γ-PGA for polysaccharide by-products, less than 1% (w/w) was detected which suggested *B. subtilis* TAM-4 to scarcely produce polysaccharide by-products. *B. subtilis* IFO3335 in contrast produces γ-PGA as well as a polysaccharide by-product during growth in a glucose containing medium (Ito *et al*., 1996). In addition, it was discovered that the ratio of D- and L- isomer of glutamic acid that makes up γ-PGA in γ-PGA from *B. subtilis* TAM-4 remains constant (78:22, D-isomer: L-isomer) throughout the cultivation period, this suggested that γ-PGA from *B. subtilis* TAM-4 was elongated without a change in the diastereoisomer ratio in the molecule (Ito *et al*., 1996).

2.6 Factors Affecting Yield of γ-PGA

2.6.1 Medium Components

L-glutamic acid carries out different functions in the different strains that produce γ-PGA. Strain and other medium components are the determining factors for the amount of L-glutamic acid added in the medium during γ-PGA production. During production of γ-PGA, L-glutamic acid showed significant interactions with other components of the medium. Therefore, its concentration has to be optimized since it directly affects the cost of production of γ-PGA because it is relatively expensive. The rate of conversion of L-glutamic acid to γ-PGA is a principal tool for the determination of the effective concentration of L-glutamic acid in the medium. Concentrations of 20-30 g/l of L- glutamic acid have been suggested by most literature for the production of γ-PGA (Bajaj and Singhal, 2011).

Citric acid is another essential component in γ-PGA production as reported by Goto and Kunioka (1992). L-glutamic acid has been reported to be produced from citric acid via isocitric and α-ketoglutaric acid in the TCA cycle and γ-PGA is polymerized from the produced glutamic acid. Carbon source selection for γ-PGA synthesis has been observed to be strain specific. In most of the strains, glucose and glycerol support γ-PGA production. γ-PGA production was enhanced when glycerol was added as co-substrate with L-glutamic acid and citric acid (Du *et al*., 2005). It was suggested that polyglutamyl synthetase which catalyses polymerization of glutamic acid to γ-PGA may be stimulated by glycerol (Bajaj and Singhal, 2011).

Wu *et al*. (2010) reported that in addition to stimulating production of γ-PGA in *B. subtilis* NX-2, the presence of glycerol in the culture medium can also reduce the molecular weight of γ-PGA. An increase in yield from 26.7g/l-31.7g/l was obtained with 20g/l glycerol and the

molecular weight could be regulated between $2.43x10^6$ Da and $1.42x10^6$ Da with glycerol concentration ranging between 0-60g/l.

In addition, the presence of glycerol in the fermentation medium is responsible for a decrease in γ-PGA chain length which in turn results in decrease of the viscosity of the fermentation broth and enhances substrates uptake. This ultimately improves cell growth and production of γ-PGA (Bajaj and Singhal, 2011).

Conversion of carbon sources into γ-PGA using ^{13}C labelled glucose has been analysed using ^{13}C NMR method. Reports of the investigation showed that glucose is the source of energy required for γ-PGA production and it was used as the growth-limiting substrate for cell growth; but L-glutamic acid was the preferred principal substrate for formation of γ-PGA (Bajaj and Singhal, 2011).

Presence of organic nitrogen sources resulted in a lower yield of γ-PGA when compared with yields in the presence of inorganic nitrogen sources (Bajaj and Singhal 2011). It has been reported that the addition of ammonium sulphate to culture medium influenced production of γ-PGA. Ammonium sulphate concentration of about 0.5g-1g was suggested as no by-product was detected at this concentration in contrast to a lower concentration of 0.25g where an increase in γ-PGA yield was recorded but by-product was detected (Goto and Kunioka, 1992).

It has also been reported that the presence of inorganic salts like $CaCl_2$ and $MnSO_4$ not only have a significant effect on yield but also on the stereochemical composition of γ-PGA (Bajaj and Singhal, 2011). Addition of $CaCl_2$ as a novel additive to enhance γ-PGA production yield in *B. subtilis* CGMCC 2108 reduced culture broth viscosity effectively and increased extracellular glutamate consumption by 11.4% which resulted in a γ-PGA yield of 9.07 g/l (Huang *et al*., 2011).

In addition, the presence of $CaCl_2$ also increased the activities of three key enzymes around the important 2-oxoglutarate branch of the biosynthetic pathway of γ-PGA: isocitrate dehydrogenase, glutamate dehydrogenase (GDH) and 2-oxoglutarate dehydrogenase complex. GDH activity was particularly increased by more than 8-fold, this suggests that more 2-oxoglutarate was channelled towards glutamate synthesis (substrate of γ-PGA). It is important to note that addition of $CaCl_2$ did not affect the molecular weight of γ-PGA (Huang *et al*., 2011).

2.6.2 Effect of Aeration, Agitation and pH on γ-PGA Production

Aeration, pH and agitation are other factors that can affect production and yield of γ-PGA.

The effects of aeration and pH were investigated by Cromwick *et al*. (1996). It was reported that to get a yield increase in γ-PGA production and to maintain high specific productivity at prolonged culture times, pH 6.5 is optimal. The report shows the metabolism of citrate to γ-PGA is essential for cultures grown at pH 6.5 against pH 5.5 and 7.4. When pH values of cultures were maintained at 5.5, 6.5 or 7.4, there was little effect on the time course changes in molecular weight of γ-PGA, distribution of γ-PGA molecular weight and stereochemistry of γ-PGA.

Improved cell growth and γ-PGA production was reported with an increase in oxygen supply to fermenters when pH was maintained at 6.5. Doubled maximum cell dry weight and rapid depletion of glycerol, L-glutamic acid and citric acid were observed and γ-PGA concentrations was up to 23g/l against 6.3g/l when oxygen content was low within a culture period of 48 hours. It was suggested that in order to achieve substantial improvements in specific productivity and γ-PGA volumetric yields, oxygen enriched air, modified impeller design and maintenance of a suitable feeding schedule of carbon and nutrients during cultivation can all be used (Cromwick *et al*., 1996).

The effect of agitation on production of γ-PGA was investigated by using a medium that contains 6% sodium L-glutamic acid. At 300 rpm or 350 rpm; γ-PGA production was low while at agitation rate of 400 rpm or 450 rpm, γ-PGA production rate was higher than at 300 rpm or 350 rpm. Maximum agitation effect appears to be at 400 rpm since at 450 rpm agitation, similar γ-PGA production yield was reported (Ogawa *et al*., 1997).

2.7 Conformation and Enantiomeric Composition of γ-PGA

Depending on the environmental conditions, γ-PGA can exhibit five different conformations: a-helix, β-sheet, helix to- random coil transition, random coil and enveloped aggregate. Factors such as pH, polymer concentration and ionic strength can change the conformational state of γ-PGA (Ho *et al*., 2006).

Investigations of the structure of poly- (γ-D- glutamic acid) in solutions have revealed different results based on the degree of ionization of the polymer. A helical conformation is revealed in the unionized state while in the ionized state, the polymer acts like it is in a random coil state. A helical conformation was revealed at low pH and low concentration of γ-PGA (0.1% w/v) while a structural shift to β-form for the deprotonated biopolymer was observed at neutral to high pH (Shih and Van, 2001).

γ-PGA assumed an elongated stiff conformation at low concentration (0.1% w/v) and at pH greater than 7. γ-PGA from *B. licheniformis* takes a helical conformation at low ionic strength and as ionic strength increases, a structural change to a mixture of helix and β-sheet at 0.1M and to predominantly β-sheet at 0.5M. Results from the concentration effect on γ-PGA conformation reveals that at low concentration, γ-PGA was in a helical conformation while in the β-conformation at a higher concentration. A change in conformation of a biopolymer can affect its physical properties as conformational change in γ-PGA can affect its ability to bind metals (Shih and Van, 2001).

γ-PGA has various enantiomeric compositions and they affect its method of extraction after fermentation. If γ-PGA contains only L or D enantiomers, then it dissolves in ethanol. However, if L and D forms are in equimolar amounts, then γ-PGA precipitates in ethanol (Candela and Fouet, 2006). Researchers have confirmed that the enantiomeric composition of γ-PGA is dependent on Mn^{2+} in both *B. licheniformis* and *B. subtilis*. The enantiomeric composition and molecular mass of γ-PGA is also largely affected by culture conditions, both of which can alter its properties (Shih and Wu, 2009).

In addition, the knowledge of the conformational state of γ-PGA is essential when it is used in for different application since it has been reported that a small change in environmental conditions can change the properties of γ-PGA to a large extent (Ho *et al.*, 2006).

2.8 Applications of γ-PGA

γ-PGA is a natural biopolymer and due to its distinct characteristics such as biodegradability, non-toxicity, solubility and edibility, it has been applied in many different biotechnology fields ranging from acting as biological adhesives, in waste water treatment as a chelating agent (Sung *et al*., 2005), in cosmetics (Ho *et al*., 2006) as a moisture container, as a stabilizer in foods and has been applied medically as a drug carrier (Sonaje *et al*., 2010) and as an osteoporosis-preventing factor (Sung *et al*., 2005). γ-PGA has also been used for probiotic bacteria as a cryoprotectant and as protective coating against the high acidic environment of the stomach (Bhat *et al.*, 2013; Bhat *et al*., 2015). The various applications of γ-PGA are presented in table 2.3

2.8.1 Application as a Cryoprotectant/Support Carrier

The antifreeze property of poly (glutamic acids) (PGAs) was investigated and a higher antifreeze activity was recorded when the molecular weight of PGAs was in the range of 20,000 than glucose which is known to have high antifreeze property (Misuiki *et al*., 1998).

Almost all substances that have high antifreeze property also possess cryoprotective effect (Mitsuiki *et al*., 1998). Bhat *et al*., (2013) investigated the cryoprotective effect of γ-PGA since its antifreeze property is well known. The study was aimed at testing the effect of γ-PGA produced by *B. subtilis* natto ATCC 15245 on the viability of probiotic bacteria (*L. paracasei, B. breve* and *B. longum*) during freeze drying. The study demonstrated that 10% γ-PGA protected *L. paracasei* better than sucrose; a well-known cryoprotectant. It also demonstrated comparable cryoprotectant activity to sucrose with *B. breve* and *B. longum*.

Bhat *et al*. (2015) also studied the survival of probiotic bacteria (*B. breve* and *B. longum*) when combined with a γ-PGA matrix during storage in fruit juices and during ingestion. Results demonstrated that the combination of probiotic bacteria with a γ-PGA matrix improved survival of *B. breve* and *B. longum* during storage in fresh orange juice over a period of 39 days. It was also reported that survival of *B. breve* and *B. longum* was improved with the γ-PGA matrix during exposure to simulated gastric juice (pH 2) for 4 hours compared with free cells, a total loss in viability was recorded after 1 hour in free cells while in γ-PGA-protected cells, an average of 8 Log CFU/ml was recorded after 4 hours of exposure to simulated gastric juice.

Gomaa (2016) reported that γ-PGA is able to improve the survival of probiotic bacteria (*L. rhamnosus, L. paracasei and L. plantarum*) during freeze drying and during storage in orange juice. It was also reported that the concentrations of citric, malic and ascorbic acids present in

the orange juice did not change which suggested γ-PGA can be used as a non-dairy delivery system for probiotic bacteria.

Table 2.3: Application of γ-PGA and its Derivatives

Field	Application	Chemical /Biochemical Functions
Medicine	Drug delivery	Paclitaxel poliglumex (PPX) a macromolecular conjugate of paclitaxel and poly-l-glutamic acid demonstrated better effect than the standard paclitaxel. Results also revealed accumulation of PPX in tumour tissue where the active ingredient paclitaxel was slowly and progressively released (Singer, 2005).
	Tissue engineering	A polyelectrolyte complex (PEC) made up of chitosan and γ-PGA was designed for wound dressing. PEC provided suitable moisture content and demonstrated excellent mechanical properties that allowed the dressings to be easily removed from the wound surface without destroying newly formed tissue (Tsao *et al*., 2011).
		The surface hydrophilicity, rate of water absorption, swelling ratio of composite biomaterial and mechanical strength of chitosan matrices has been improved by modification with γ-PGA (Bajaj and Singhal, 2011).
	Biological adhesives	A hydrogel glue produced when gelatine and poly (L-glutamic acid) were mixed with a water soluble carbodiimide demonstrated a superior sealing effect of the lung air leak than the conventional fibrin glue (Otani *et al*., 1999).
	Metal chelator	Super paramagnetic iron oxide nanoparticles coated with γ-PGA exhibited high heavy metal removal capabilities from simulated gastrointestinal fluid and a meal solution (Inbaraj and Chen, 2012).
Food	Cryoprotectant	γ-PGA sodium salt produced by *B. licheniformis* has antifreeze activity and is an effective cryoprotectants for frozen foods due to its weaker taste and it can be added to food in large quantity without altering the taste of the food (Najar and Das, 2015).
	Bitterness relieving agent	γ-PGA can be used to relief the bitter taste of amino acids, peptides, caffeine, quinines, etc. in food (Shih and Van, 2001).
	Texture enhancer	γ-PGA can be used to improve the texture of starch-based baked products, noodles and as ice-cream stabilizer (Najar and Das, 2015).
	Osteoporosis preventing agent	γ-PGA is able to dissolve calcium and magnesium compounds and in turn form stable coordinated stable ionic complexes. This will increase the bioavailability of calcium and magnesium hence reducing old-age osteoporosis conditions and as well as improve the formation of healthy bones. (Ho *et al*., 2006).
	Mends taste and drinkability of certain drinks	Low concentrations of γ-PGA have been reported to mend the taste and drinkability of juices and other drinks (Najar and Das, 2015).

Table 2.3 Continued

Field	Application	Chemical /Biochemical Functions
Cosmetics	Moisturiser	The use of moisturisers containing γ-PGA (Na^+ form) and γ-polyglutamate hydrogels (Na^+ form) was examined and results showed that moisturisers containing γ-PGA were better for the improvement of skin elasticity (Ho *et al*., 2006).
	Increases hair strength	γ-PGA has been reported to be able to increase hair strength and to allow hair to withstand bleaching process by increasing its ability to retain moisture and by forming a barrier that is able to dilute the chemical interactions of the colourings applied with the protein contents of the hair (Bajaj and Singhal 2011).
Treatment of waste water	Removal of heavy metals	γ-PGA functionalized cellulosic membranes were tested on water waste that contained lead and a minimum of 380-fold volume reduction can be attained through membrane encapsulation (Shih and Van, 2001).
	Removal of dye	γ-PGA can be used for the effective removal of basic dyes from aqueous solution. It was reported that at pH 1.0; 98% of the dye absorbed in γ-PGA can be recovered; this enhances reuse of spent γ-PGA (Inbaraj *et al*., 2006b).
	Biopolymer flocculant	The flocculation properties of γ-PGA from *B. subtilis* was investigated and it was reported that γ-PGA possesses flocculating activity in various organic and inorganic suspensions (Yokoi *et al.*, 1996).
Agriculture	Animal feed additives	Absorption of minerals is promoted by γ-PGA (Najar and Das, 2015) and it also increases the strength of egg-shells (Luo *et al*., 2016).
	Biocontrol agent and fertilizer synergist	γ-PGA obtained from the fermentation of *B. subtilis* strain B6-1 and soybean and sweet potato residues effectively supressed cucumber wilts and also increased the dry weights of roots and shoots of cucumber seedlings (Wang *et al*., 2008).
Others	Biodegradable plastics	γ-PGA can be used as a substitute for chemically synthesised non-biodegradable plastics (Shih and Van, 2009).
		A complex made up of L-PGA and hexadecylpyridinium cation (HDP^+) has been reported to be able to form thermoplastics. HDP^+ acts to suppress the hydrophilicity of γ-PGA and the complex formed can be moulded into various shapes and sizes by pressurization (Najar and Das, 2015).

Table 2.3 Continued

Field	Application	Chemical /Biochemical Functions
	Antibacterial activity	It has been reported that magnetite nanoparticles (MNPs) that are modified with the sodium (NaPGA) and calcium (CaPGA) salts of γ-PGA are cytocompatible and possess antibacterial activity *in vitro* against *Salmonella enteritidis* (SE01) than the commercial antibiotics linezolid and cefaclor. CaPGA was effective against *E. coli* 0157:H7 TWC01 while Na PGA was effective against *E. coli* ATCC 8739 and *S. aureus* ATCC 10832 (Inbaraj *et al*., 2011).
	Treatment of xerostomia (dry mouth)	A sialagogue made up of γ-PGA or its salt if blended into an oral suspension and food product is able to make the oral mucosal pleasant even in severe cases of xerostomia (Uotani *et al*., 2011).
	Gene delivery	A γ-PGA complex (pDNA/PEI/γ-PGA) was developed and it was reported to be a useful gene delivery system with low toxicity and high transfection efficiency (Kurosaki *et al*., 2009).
	Glucose sensor	Fine needle-type glucose sensors were made using γ-PGA as a glucose oxidase and a permselective inner membrane. The presence of electroactive compounds such as ascorbic acids and uric acids had no effect on the obtained glucose sensor. The glucose sensor also provided long term stability of about one month. γ-PGA was reported to function as a sufficient perselective film material (Yasuzawa *et al*.,2011).

3.0. INTRODUCTION TO BACTERIAL CELLULOSE

3.1 Background

Cellulose is the most abundant organic biopolymer in nature (Keshk, 2014; Son *et al*., 2002); the major polymeric component of plant matter (Bayer *et al*., 1998), commonly harvested from trees and cotton (Mohite and Patil, 2013) is almost inexhaustible (Klemm *et al*., 2001).

Cellulose is of extreme economic importance worldwide. It is the major constituent of cotton, making over 94% of cotton and 50% of wood. Together with other cellulose derivatives such as cellophane, rayon and cellulose acetate; cotton and wood are the major resources for all cellulose products such as paper, textiles, construction material and cardboard (Keshk, 2014).

Cellulose obtained from land and forest plants is derived from glucose that is produced via photosynthesis in living plant cells. The same type of carbon dioxide fixation present in photosynthesis of land plants is used by unicellular plankton or algae in the oceans for cellulose synthesis. These organisms are believed to be nature's largest resource for cellulose synthesis. Some fungi and bacteria that lack photosynthetic capacity are able to synthesize cellulose from glucose or other organic substrates (Keshk, 2014).

Cellulose is a homopolymer that is made up of glucose monomers linked together by β (1-4) glycosidic linkages with chemical formula $(C_6H_{10}O_5)_n$ (Brown, 1987; Sheykhnazari *et al*., 2011).

Bacterial cellulose (BC) was described as "*a sort of moist skin, swollen, gelatinous and slippery..."* (Iguchi *et al*., 2000). A gelatinous mat formed on the surface of the broth during the fermentation of vinegar was identified as equivalent chemically to cell-wall cellulose and upon microscopic examination, the bacteria *Gluconacetobacter xylinus* (formally known as *Acetobacter xylinum*, now known as *Komagataeibacter xylinus*, it will be referred to as

Gluconacetobacter xylinus throughout this work) was found distributed around the matrix (Klemm *et al*., 2001).

Gupta *et al*. (2016) defined BC as a biosynthetic homopolymer synthesised by the Gram negative obligate aerobe *Gluconacetobacter xylinus*. It is produced in the form of pellicles at the air-liquid interface of the culture medium in a static environment (Klemm *et al*., 2001; Krystynowicz *et al*., 2002), while in agitated culture conditions, bacterial cellulose is produced in the form of a fibrous suspension, irregular masses, pellets or spheres (Krystynowicz *et al*., 2002).

BC is in the form of microfibrils that are made up of glucan chains interlocked by hydrogen bonds in order for a crystalline domain to be produced (Chawla *et al*., 2009).

3.2 Microoganisms Producing Cellulose

Microorganisms such as bacteria, algae and fungi are found to produce cellulose (Chawla *et al*., 2009). Gram-positive *Sarcina ventriculi* also synthesises cellulose and cellulose accounts for about 15% of the total dry cell mass. Gram-negative species in the genera *Agrobacterium, Achromobacter, Aerobacter, Azotobacter, Rhizobium, Pseudomonas, Salmonella, Escherichia, Alcaligenes* and *Gluconacetobacter* are able to produce cellulose (Chawla *et al*., 2009; Mohite and Patil, 2013; Sheykhnazari *et al*., 2011). When the tumour-forming *Agrobacterium tumefaciens* bacterium gains contact with host plant cells, it secretes cellulose fibrils from all sides of the cell, a process that supports virulence and enhances cell attachment. *Gluconeacetobacter xylinus, G. hansenii* and *G. pasteurianus* are the most effective producers of cellulose (Chawla *et al*., 2009). *Gluconacetobacter* (vinegar bacterium), is able to use glucose, sugar, glycerol or other organic substrates and convert them into pure cellulose (Keshk, 2014).

Gluconacetobacter xylinus is mostly used and studied for bacterial cellulose synthesis. *G. xylinus* is a Gram-negative, strictly aerobic rod-like bacterium with unusual acid tolerance that is able to survive at pH below 5.0 (Klemm *et al*., 2001). It produces cellulose at pH between 3-7 and at temperatures between 25 °C and 30 °C (Sheykhnazari *et al*., 2011). They are highly ubiquitous, actively motile and are mostly found in areas where sugars and plant carbohydrate fermentation take place e.g. on damaged fruits, on flowers and in unpasteurised beer, juice and wine (Klemm *et al*., 2001).

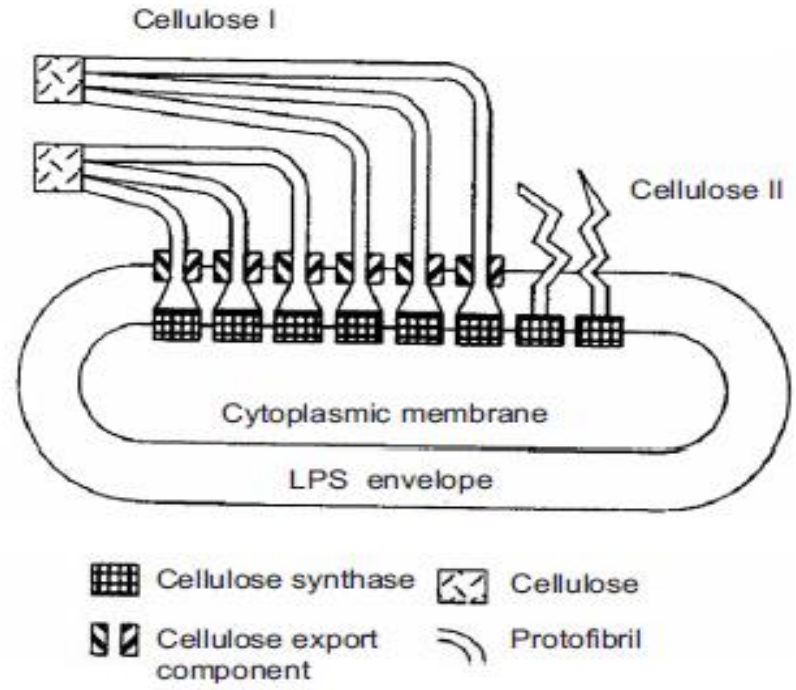


Figure 3.1: Assembly of cellulose microfibrils by *G. xylinus* (adapted from Esa *et al*., 2014)

Glucose, sucrose, and mannitol are examples of carbon sources that are used by *G. xylinus* for cellulose synthesis (Sheykhnazari *et al*., 2011). Electron microscope observations have revealed that the cellulose produced by *G. xylinus* exists in the form of fibres. A structurally

homogenous slimy substance is first secreted by the bacteria followed by the formation of cellulose fibres (Chawla *et al*., 2009).

Two forms of cellulose are formed by *G. xylinus:* cellulose I which is a ribbon-like polymer and cellulose II which is the thermodynamically more stable amorphous polymer. Figure 3.1 shows the differences in the assembly of the two different types of cellulose formed by *G. xylinus* outside the cytoplasmic membrane (Chawla *et al*. 2009).

Most of the unique properties exhibited by bacterial cellulose are as a result of its microbibrillar structure (Chawla *et al*., 2009).

3.3 Differences between Plant Cellulose and Bacterial Cellulose

Cellulose produced by plants and bacteria have the same molecular formula $(C_6H_{10}O_5)_n$ in which "n" can be within the range of several hundreds to thousands. The chemical structure is given in figure 3.2.

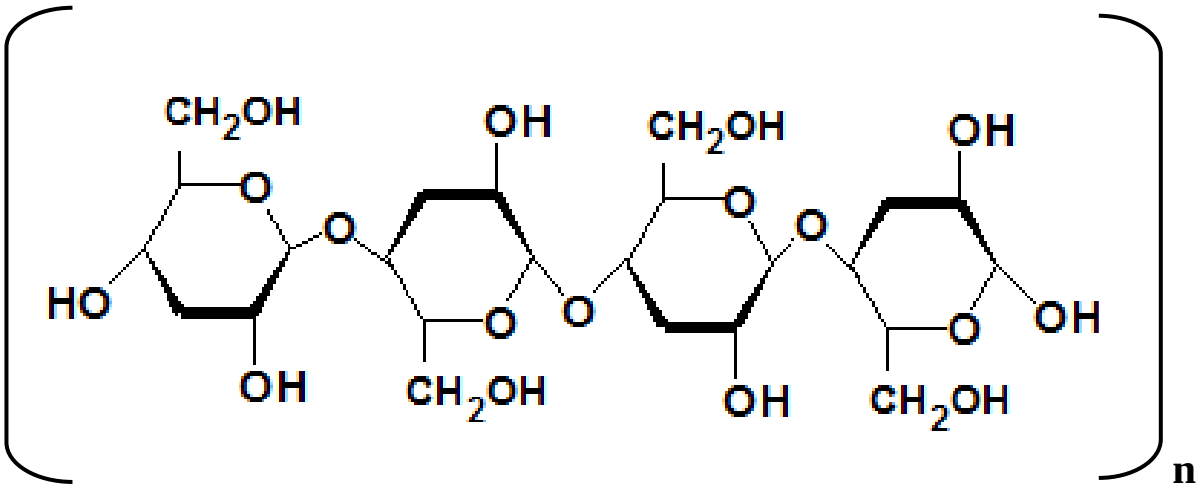


Figure 3.2: Structure of cellulose. (n100- ~10,000) (adapted from Alghazzawi, 2015)

Several cellulose chains accumulate and form microfibrils in plant cellulose. These microfibrils then form bundles and a cluster of high-order structure called fibril lamella and

fibre cells. Plant cellulose is found in the cell wall and forms a complex structure with hemi cellulose, lignin and other impurities, in contrast, BC is produced in the form of ribbon made up of bundles of microfibrils. These ribbons are usually very thin with a width of about one-hundredth of that of plant cellulose, they then grow as visible reticular structures and form regular structures (Shoda and Sugano, 2005). BC is pure and devoid of contaminants such as lignin, pectin and hemicellulose, which are present in plant cellulose (Gupta *et al*., 2016).

Other major differences between plant cellulose and bacterial cellulose are highlighted in table 3.1, while figure 3.3 shows an electron micrograph from plant cellulose and BC.

3.4 PROPERTIES OF BACTERIAL CELLULOSE

3.4.1 Morphology

The gelatinous bacterial cellulose pellicle produced in a static culture is about 99% water and has a 3D ultrafine fibrous network structure. The ribbon-shaped fibrils are less than 100nm wide and are made up of elementary nano fibrils that are assembled in bundles with lateral size of 7-8nm (Andrade *et al*., 2010). The nanofibrillar structure of bacterial cellulose is responsible for most of its properties such as high tensile strength, higher degree of polymerization and crystallinity index (Ashjaran *et al*., 2013).

The 3D structure of BC results in high cellulose crystallinity with the range of 60% - 90% (Ashjaran *et al*., 2013). Cellulose has several crystalline polymorphisms cellulose I, II, III and IV. Cellulose I is the crystalline cellulose and the most predominant allomorph and BC is made up of mostly cellulose I. Cellulose I which can be converted to cellulose II or III is known to have a crystalline structure which is a mixture of two distinct crystalline variations: meta-stable state cellulose I_α (60%) and stable state cellulose I_β (40%). Bacterial cellulose has high

Table 3.1: Differences between bacterial cellulose and plant cellulose

BACTERIAL CELLULOSE	PLANT CELLULOSE
Cellulose fibril product is about 100 times thinner than that of plant cellulose (Chawla *et al*., 2009).	Cellulose fibrils in plants make up the integral part of the complex polysaccharide cell wall matrix (Ross *et al*., 1991)
Do not contain impurities, not mixed with non-cellulosic polysaccharides or other biogenic products (Andrade *et al*., 2010; Klemm *et al*., 2001; Sheykhnazari *et al*., 2011).	Impurities such as hemicellulose, pectin, lignin and other biogenic products are present (Sheykhnazari *et al*., 2011; Klemm *et al*., 2001).
Exhibit higher crystallinity (80%-90%), high water-holding capacity, high mechanical strength in the wet state and can be sterilised without changes to its structures and properties (Amin *et al*., 2012; Son *et al*., 2003).	The crystallinity, water-holding capacity, and mechanical strength not as high as cellulose from bacteria (Sheykhnazari *et al*., 2011; Klemm *et al*., 2001).
Exhibit excellent biodegradability and excellent biological affinity (Hungund and Gupta, 2010)	Do not exhibit excellent biodegradability and biological affinity (Hungund and Gupta, 2010)
Exhibits ultra-fine nanofiber network structure about 3.5 nm width (Amin *et al*., *2012;* Klemm *et al*., 2001)	Do not exhibit ultra-fine network structure (Klemm *et al*., 2001)
Mouldable *in-situ* and is highly available in the initial wet state (Klemm *et al*., 2001)	Not mouldable *in-situ* and not available in the initial wet state (Klemm *et al*., 2001)
Do not have intracellular cavity (Kurosumi *et al*., 2009).	Possess intracellular cavity (Kurosumi *et al*., 2009)
Degree of polymerization is 4000-6000 (Andrade *et al*., 2010)	Degree of polymerization is 13000-14000 (Andrade *et al*., 2010).
Uridine Diphosphoglucose (UDPG) is a major intermediate in the biosynthesis of bacterial cellulose (Keshk, 2014)	Guanidine Diphosphoglucose (GDPG) is required for the biosynthesis of cellulose from plants (Keshk, 2014).

surface area and good mechanical strength because of its crystallinity and small fibre diameter (Bi *et al*., 2014; Roushdy, 2015). Morphology and structural characteristics of BC can be affected by different factors such as cultivation method and bacterial strain (Ul-Islam *et al*., 2013; Bi *et al*., 2014).

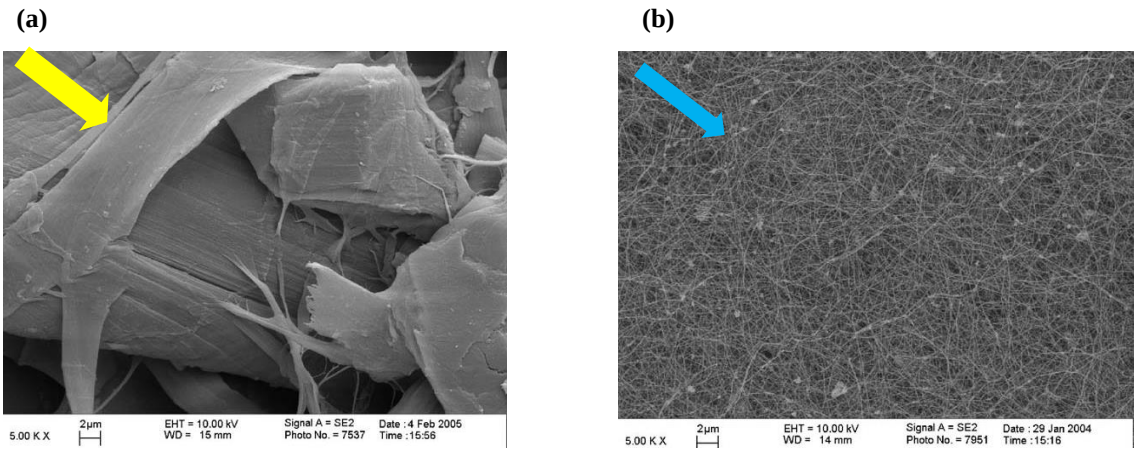


Figure 3.3: Electron micrographs of fibres from (a) common pulp from plant cellulose and (b) bacterial cellulose (Yellow arrow shows the large fibre of plant cellulose while blue arrow shows the tiny microfibril of BC(Czaja *et al*., 2006)

Bi *et al*. (2014) investigated the effect of different strains on the morphology and structural characteristics of BC. Different types of BCs including flocky asterisk-like BC (*G. entanii* ACCC10215) and solid sphere-like BC by (*Gluconacetobacter nataicola* Y19) were produced in HS medium. They observed that asterisk-like BCs had larger pores than the solid sphere-like BCs as revealed by SEM. In an agitated environment, the BC microfibrils were curlier and more twisted than in a stationary environment. Cellulose synthesized by *G. xylinus* has crystalline structure, enterobacteria appear to produce mostly non-crystalline (amorphous) cellulose (Romling and Galperin, 2015).

The biological role and application of bacterial cellulose are based on the uniqueness of the fibre morphology (Andrade *et al*., 2010).

3.4.2 Mechanical Properties of Bacterial Cellulose

Both treatment after synthesis and growth culture environment affect the macro structure and micro structure of bacterial cellulose (Andrade *et al*., 2010). Thus, after 7 days of culture, a

bacterial cellulose pellicle obtained and air-dried at low pressure and at 20 °C showed a Young's modulus of 16.9 GPa, tensile strength of 256 MPa and 1.7% elongation, however, when BC pellicle was dried with application of excess pressure of between 490 kPa -1960 kPa through the heat-press method, tensile strength and elongation reduced while the Young's modulus remained constant (Iguchi *et al*., 2000).

A bacterial pellicle dried after 48 h of culture showed a tensile strength of 5.21MPa and a break strain of 3.75%. The high planar orientation of ribbons pressed into a sheet, high crystallinity, complex network of the ribbons and ultrafine structure appear to be the basis of the high Young's modulus and tensile strength of bacterial cellulose pellicle (Andrade *et al*., 2010).

3.4.3 Water Holding Capacity

Being hydrophilic, bacterial cellulose has the ability to retain over 700 times its dry weight in water (Moosavi-Nasab and Yousefi, 2011). The presence of pore structure within wet BC pellicles explains its hydrophilicity and this is dependent on the extensive interior surface area of the interstitial spaces of the wet BC matrix. Water retention values (WRV) in the range of about 1000% has been reported in wet BC and this reduces tremendously after air-drying at 100°C. Freeze drying has been suggested as the best drying method to preserve the porous structure of bacterial cellulose (Klemm *et al*., 2001).

3.4.4 Permeability

Investigations were conducted into characterising the transport of vitamin B12, lysozyme and bovine serum albumin with molecular weight: 1355 Da, 14.3 kDa and 66.3 kDa respectively through hydrated BC, with vitamin B12 as the low molecular weight marker molecule. Results from the experiments showed a double transport mechanism of the solute via the

continuous water phase and cellulose matrix in addition to hindrance of molecular diffusion through fibre blockage. SEM images also revealed the non-existence of cylindrical pores but micro-channels of different sizes through which solute diffusion occurs (Sokolnicki *et al*., 2006).

3.5 Bacterial Cellulose Synthesis

3.5.1 Genes Involved in Bacterial Cellulose Synthesis

It has been established that *G. xylinus, Agrobacterium tumefaciens, Rhizobium leguminosarum bv. trifolii, Sarcina ventriculi, Salmonella spp*., *Escherichia coli, Klebsiella pneumoniae* and several species of *Cyanobacteria* produce cellulose. In all the mentioned organisms with the exception of *S. ventriculi*, a cellulose synthase has been identified (Romling, 2002).

A four-gene *bcsABCD* (*acsABCD*) operon called *bcs or acs* (bacteria cellulose synthase or acetobacter cellulose synthase) involved in cellulose bio-synthesis was initially identified in *Gluconacetobacter* (*Acetobacter*) *xylinus* (Romling *et al*., 2015; Deng *et al*., 2013). Although, all four proteins were required for maximal cellulose production *in vivo*, however, the products of the first two genes, *bcsA* and *bcsB* were essential for the BCS activity *in vitro* showing that *bcsC* and *bcsD* were involved in exporting the glucan molecules and packing them at the cell surface. Three more genes were included on *K. xylinus bcs* locus namely *bcsZ*, *ccpA* (upstream of *bcsABCD* genes) and *bglX* (downstream of *bcsABCD* genes). An endoglucanase and a β-glucosidase are the products of *bcsZ* and *bglX* respectively while the product of the *ccpA* (cellulose complementing factor) gene was required for cellulose production (Sunagawa *et al*., 2013; Romling and Galperin, 2015).

Cellulose synthase encoded by *bcsA* also called *acsA* or *celA* is located in the cytoplasmic membrane and *bcsA* is considered to be the catalytic subunit for cellulose biosynthesis. The second gene is for the C-di-GMP binding protein and is encoded by *bcsB* (*acsB, celB*). The *bcsA* gene and *bcsB* gene in *G. xylinus* are occasionally joined to a single ORF in type II cellulose synthase which shows the tight functional coupling of the two protein products for which partial closeness has been demonstrated (Römling, 2002).

It has been suggested that there is variability in the position and in the requirement of additional genes for cellulose biosynthesis. *BcsZ* (CMC in *G. xylinus*) has been demonstrated to encode a cellulase that is required for cellulose synthesis. All cellulose producing species have been reported to possess the *bcsZ* gene and is encoded by the cellulose biosynthesis operons of enterobacterial species, *A. tumifaciens* and *R. leguminosarum bv. trifolii*, located outside but adjacent to the cellulose biosynthesis operon in several *G. xylinus* strains. The *BcsC* required *in vivo* for cellulose synthesis is present in enterobacterial species, the pseudomonads and *G. xylinus* (Römling, 2002).

3.5.2 Mechanism of Bacterial Cellulose Synthesis

The unidirectional polarity of cellulose fibrils and their regular but variable width must be considered when thinking of the modes of biosynthesis of cellulose. Almost all cellulose fibrils isolated from algae and plants bear the crystalline unit structures which are laterally and unidirectionally aligned, this is expected of β-1→4 glucan chains. This parallel arrangement is termed cellulose I, cellulose II, which is the second form of cellulose produced is the crystalline fibrils that bear the antiparallel polyglucan chains. This occurs when partially solubilised native cellulose reassembles into the thermodynamically stable form (Ross *et al*., 1991).

In *Gluconacetobacter xylinus*, the production of bacterial cellulose takes place between the outer membrane and cytoplasma membrane by a cellulose-synthesising complex. The terminal complexes (TC) or the cellulose synthesising complexes are arranged linearly and are in association with pores present at the surface of the bacterium (Jonas and Farah 1997 and Klemm *et al*., 2001).

The biochemical pathway for cellulose synthesis from glucose is linked to cell growth and to cellulose formation (Jonas and Farah, 1998). In *G. xylinus*, the synthesis of cellulose is closely linked with catabolic processes of oxidation; about 10% of energy acquired from the catabolic processes is used up during cellulose synthesis. Anabolic processes such as protein synthesis are not affected by cellulose synthesis. The biosynthetic pathway for bacterial cellulose production is a specifically regulated process which involves individual enzymes as well as complexes of regulatory and catalytic proteins as shown in figure 3.4 (Chawla *et al*., 2009).

Carbon compounds such as glycerol, dihydroxyacetone, hexoses, dicarboxylic acids and pyruvate are converted to cellulose by *G. xylinus* with approximately 50% efficiency. Dicarboxylic acids and pyruvate go into the Krebs cycle and as a result of decarboxylation of oxaloacetate to pyruvate are converted to hexoses through gluconeogenesis; this process is similar for glycerol, dihydroxyacetone and intermediates of pentose phosphate pathway. UDP-glucose the direct precursor of cellulose is a product of a conventional pathway which involves different series of enzyme catalysed reactions depicted in figure 3.4 (Chawla *et al*., 2009).

The synthesis of cellulose in plants and bacteria producing cellulose follows two intermediate steps: Formation of β-1→4 glucan chain with glucose unit polymerization and crystallization

and assembly of cellulose chain. The rate of assembly and crystallization of cellulose limits the rate of polymerization (Chawla *et al*., 2009).

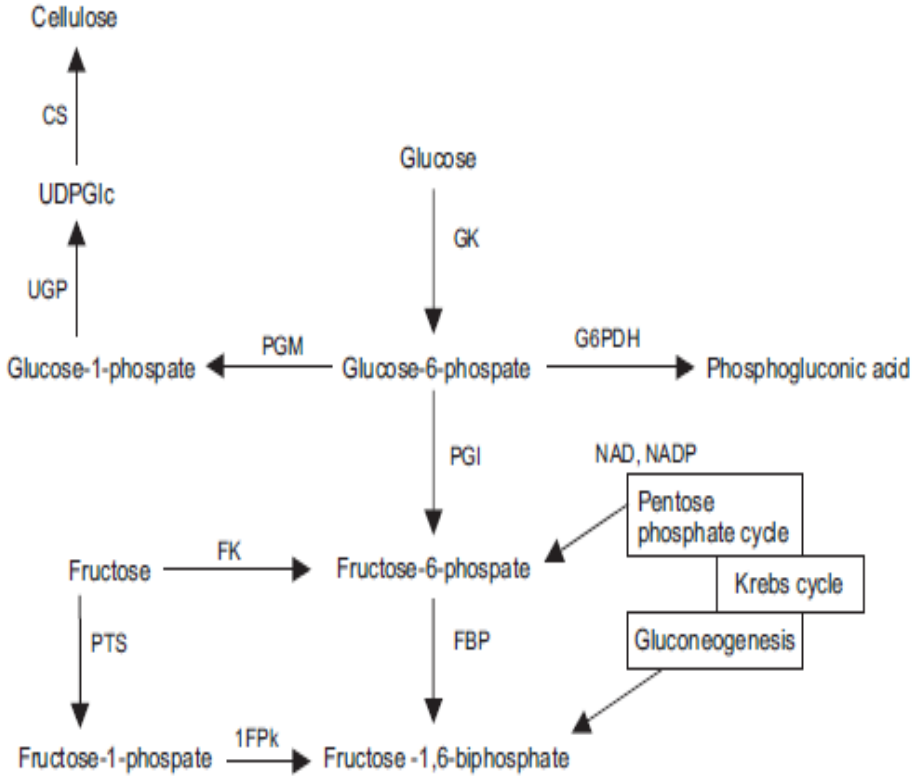


Figure 3.4: Biosynthetic pathway for synthesis of cellulose by *Gluconacetobacter xylinus.*

CS: cellulose synthase; GK: glucokinase; PGM: Phosphoglucomutase; PGI: phosphoglucoisomerase; FBP: fructose-1, 6-biphosphate phosphatase; 1FPK: Fructose-1-phosphatekinase; FK: fructokinase; UGP: pyrophosphorylase uridine diphosphoglucose; UDP Glc: uridine diphosphoglucose; G6PDH: glucose-6-phosphate dehydrogenase; NAD: nicotinamide adenine dinucleotide; NADP: nicotinamide adenine dinucleotide phosphate; PTS: system of phosphotransferases (Chawla *et al*., 2009).

3.5.2.1 Formation of β-1→4 Glucan Chain

Cellulose synthase catalyses the conversion of UDP-glucose to cellulose, this enzyme is the most important in the formation of cellulose from glucose (Andrade *et al*., 2010; Klemm *et al*., 2001). After the formation of UDP- glucose which is the precursor in cellulose synthesis,

polymerization of glucose into β-1→4 glucan chain and the elongation of these glucan chain aggregates (about 6-8 glucan chains) from the complex takes place, this forms ribbon-like structure of cellulose chains formed by a lot of individual cellulose chains. Cellulose biosynthesis is catalysed by cellulose synthase through the polymerization of the glucose units into the β-1→4 glucan chain. In *G. xylinus,* two hypotheses have been reported for this mechanism (Chawla *et al*., 2009).

The first hypothesis is that a lipid intermediate is not involved in the β-1→4 glucan polymerization. The hypothesis also assumes that the glucose residues were added to the non-reducing end of the polysaccharide while the reducing ends were sited away from the cells and were nascent polymer chains. In cellulose molecules, the torsion angle between the two adjacent glucose residues is 180°. The second hypothesis assumes that a lipid intermediate is involved in the polymerization of β-1→4 glucan. It has been proven that the lipid intermediate is involved in the synthesis of acetan, a soluble polysaccharide (Chawla *et al*., 2009).

Iron de Ianino *et al*. (1987) reported the involvement of lipid intermediate during the screening of several strains of *G. xylinus* for *in vivo* cellulose and acetan production, and for *in vitro* synthesis of a prenyl-diphosphate-hexasaccharide, using UDP-Glc, UDP-GlcA and GDP-Man as sugar donors. The lipid-bound saccharide was reportedly synthesized only by acetan producing strains

3.5.2.2 Assembly and Crystallisation of Cellulose Chain

Research has been extensively carried out on the formation and structure of cellulose microfibril and it is believed that cellulose molecules synthesised within the cells are released out of cellulose export components to form a protofibril of about 2-4nm diameter, the

protofibrils are arranged in the form of a ribbon-shaped microfibril of about 80x4 nm (Iguchi *et al*., 2000).

The presence of about 50-80 pore-like sites have been revealed by electron micrographs of the surface of the cell envelope. These sites are arranged along the long axis of the cell in a regular row and are combined with the extracellular cellulosic ribbon. The lipopolysaccharide layer's discrete structures are assumed to be the location of extrusion for precellulosic polymers. The assembly and crystallization of cellulose occurs in the extracellular space, the mutual orientation and relation of glucan chain, aggregates, microfibrils, bundles and ribbon are governed by the original pattern of extrusion sites, hence the description of the process as cell directed (Chawla *et al*., 2009).

Figure 3.1 shows the differences in the assembly of cellulose I and cellulose II. The 2μm/min typical chain elongation rate corresponds to the polymerization of more than 10^8 glucose molecules in the β-1→4 glucan (Chawla *et al*., 2009).

3.5.2.3 Biodegradation of Bacterial Cellulose

Several types of cooperating enzymes are required for effective digestion of cellulose. The conversion of cellulose to glucose requires three categories of enzymes:

1. Endo (1,4)-β-D glucanases (EC3.2.1.4): these cut the cellulose chains at random
2. Cellobiohydrolases (CBH) (EC 3.2.1.91): these enzymes cleave cellobiosyl units from the cellulose chain ends
3. β-glucosidases (EC 3.2.1.21): these convert cellobiose and soluble cellodextrins into glucose

CBH seem to be the most important group of enzymes for the degradation of native crystalline cellulose. The activities of the different enzymes are assumed to be the reason for

synergistic effects as the enzymatic activity of a combination of two or several enzymes is notably higher than the sum of the activity of the individual enzymes (Boisset *et al*., 2000).

3.6 Fermentative Production of Bacterial Cellulose

The effectiveness of bacterial cellulose production is determined by: the type of bacterial strain, type of support material and the surface structure area of the fermentation system; the culture medium components which include the carbon source, the nitrogen source, concentration of the carbon and nitrogen sources, temperature and pH of the medium. Also, of high importance is the continuous supply of oxygen and carbon source. The bacterial strain used for the production of bacterial cellulose is also important and plays a crucial role in the microstructure and rate of production of bacterial cellulose (Klemm *et al*., 2001; Andrade *et al*., 2010).

Bacterial strains that have been studied for the production of cellulose include: *Gluconacetobacter, Rhizobium, Agrobacterium, Rhodobacter and Sarcina*. *Gluconacetobacter xylinus* is the most investigated strain for the production of cellulose (Moosavi-Nasab and Yousefi, 2011; Bi *et al*., 2014; Cacisedo *et al*., 2016).

Fermentative production of BC has been performed using the defined Hestrin and Shramm medium which contains glucose, peptone, yeast extract, disodium phosphate, citric acid and adjusted to pH 6.0, however, modification of the medium components by changing the carbon sources, nitrogen source or pH has enhanced BC productivity.

Growth of bacteria statically in medium results in the production of cellulose pellicle floating in the air-liquid interface of the medium (Figure 3.5a). Under agitation at the same temperature of between 25 °C - 30 °C, the bacteria grow faster, cellulose produced under this condition appear as ball-shaped particles (figure 3.5b) (Lin *et al*., 2013; Bi *et al*., 2014). Apart from physical difference in shape, bacterial cellulose produced under agitation has a

lower polymerization degree and a lower crystallinity and cellulose I alpha content. Cellulose produced under agitation and static also differ in crystal and molecular chains structure however, the fibrillary network remains the same (Andrade *et al*., 2010; Esa *et al*., 2014).

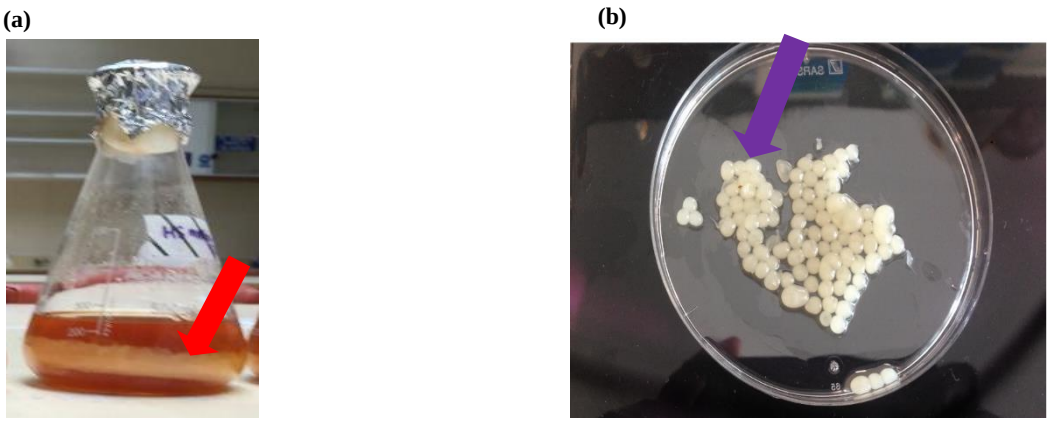


Figure 3.5: (a) BC pellicle (red arrow) produced statically by *G. xylinus* in the air-liquid interface of culture medium (b) BC pellets (purple arrow) produced by *G. xylinus* in an agitated medium (Photo credit: Olajumoke Adebayo)

Growth medium, environmental conditions and formation of by-products are general factors that affect production of cellulose. Medium that contains high carbon content to limiting nutrient ratio (most time Nitrogen) is favourable for production of polysaccharide. Required nutrients for the growth of microorganisms include nitrogen, carbon, phosphorus, sulphur, potassium and magnesium salts (Chawla *et al*., 2009).

3.6.1 Factors Affecting Production of Bacterial Cellulose

Carbon source, nitrogen source, pH, temperature and surface area of the fermentation system are factors that can affect BC production and also affect the membrane properties. Differences in bacterial strains affect the microstructure and the production rate of bacterial cellulose.

Bacterial cellulose produced in static cultures and agitated cultures differ structurally. They have the same fibril network and they differ in crystals and molecular chains structure. The degree of polymerization, crystallinity and cellulose I alpha content is lower in bacterial cellulose obtained from agitated culture than in bacterial cellulose obtained from static culture (Andrade *et al*., 2010).

3.6.1.1 Influence of Medium Components on BC Production

Glucose and sucrose are normally used as carbon source for BC production. Initial glucose concentration is of utmost importance in production of cellulose, as the by-product gluconic acid formed during cellulose production reduces the pH of the culture media and ultimately reduces cellulose production (Chawla *et al*., 2009). Initial glucose concentration of 6 g/l, 12 g/l, 24 g/l and 48 g/l for cellulose production were investigated and glucose consumption was discovered to be 100%, 100%, 68% and 28% of the initial concentration respectively (Masaoka *et al*., 1993).

Formation of gluconic acid as a by-product is the major challenge in the use of glucose as carbon source. Gluconic acid decreases the pH of the medium and ultimately decreases BC production (Zahan *et al*., 2015b). The effect of lignosulphonate on the structure and production of cellulose and the formation of gluconic acid was studied (Keshk and Sameshima, 2006a). Bacterial cellulose production was discovered to increase while gluconic acid formation decreased with the presence of lignosulphonate. FTIR results also showed that cellulose produced in the presence of lignosulphonate showed higher crystallinity index and I_{α}-rich cellulose. The increase in productivity is as a result of the presence of antioxidant and polyphenolic compounds present in lignosulphonate. The antioxidant and polyphenolic compounds inhibit the formation of gluconic acid (Keshk and Sameshima, 2006a).

The effect of different carbon sources (glucose, mannitol, sucrose, date syrup and food grade sucrose) on BC production from *G. xylinus* was studied and the obtained BCs were compared based on production yield, morphology and structure of BC. Results obtained from this study showed that BC yield was low when date syrup and food-grade sucrose were used as carbon sources while mannitol and sucrose were able to produce more BC. XRD results show that Mannitol-based produced BC, had the highest crystallinity compared to other carbon sources (Mohammadkazemi *et al*., 2015).

The effect of using the ingredients in sugar cane molasses for bacterial cellulose production by *G. xylinus* has been studied. Sugar cane molasses is made up of different components which include sucrose, glucose, fructose, nitrogenous compounds, non-nitrogenous acids, nucleic acids, vitamins, other carbohydrates, minerals and black colour substance. These components were added individually or in combination to HS medium. The results revealed an increase of 210%, 193%, 186%, 160%, and 255% with the addition of vitamins, amino acids, other carbohydrates, minerals and black colour substances in the molasses respectively in HS medium with combination of fructose and sucrose as carbon source. It was suggested that the black colour substance was the most effective component to increase bacterial cellulose yield (Premjet *et al*., 2006).

BC production has also been investigated using agricultural and industrial waste residues such as fruit wastes (apple waste), wheat straw, waste glycerol, cotton-based waste textiles and waste beer yeast as carbon sources (Lin *et al*., 2014; Li *et al*., 2015).

Nitrogen is another important media component for bacterial cellulose production. It has been reported that BC production can be enhanced with the appropriate nitrogen sources (Ramana *et al*., 2000).

Yeast extract is one of the nitrogen sources used in BC production and its effect on cell growth and BC yield has been studied. Yeast extract was added to the culture medium in the range of 5-60 g/l in the presence of 20 g/l carbon source. Maximum cellulose yield of 6.6 g/l was recorded when 40 g/l yeast extract was added to culture medium (Chawla *et al*., 2009).

El-Saied and co-workers (2008) investigated the effect of using carbon and corn steep liquor (CSL) for BC production to replace the more expensive ones that are currently in use. A yield of 1.045g/l was recorded when CSL was used as nitrogen source compared to < 1g/l obtained from other media under investigation.

3.6.1.2 Effects of pH, Temperature and Dissolved Oxygen on BC Production

Investigations have revealed that the optimum pH for BC production is between 4.0 and 6.0. At pH below 4.0, reduction in BC yield is observed. During BC production, an increase in total acid was observed and this was measured by the presence of acetic acid which was secreted during cell growth and cellulose production. This is due to the fact that *G. xylinus* is able to convert carbohydrates to acetic acid by synthesising and extruding cellulose fibres (Kongruang, 2008).

Its metabolism which is respiratory allows it to oxidise ethanol to acetic acid and to convert glucose to gluconic acid. Acetic acid, a by-product of cellulose; gluconic acid and lactic acid are the causative agents for the decrease in pH of the culture medium. Reduction in pH affects cell growth as well as cellulose production (Kongruang, 2008; Zahan *et al*., 2015a).

The optimal pH is difficult to auto regulate through the use of a pH sensor because the viscous broth sometimes gets attached to the pH sensor and lead to inadequate pH readings However, BC production in a well-buffered medium can slow-down the pH decrease caused by the accumulation of gluconic acid thereby improving BC productivity (Kuo *et al*., 2016).

Temperature is an important factor that affects cell growth and cellulose production. The optimal temperature that has been reported by majority of investigations is between 28 °C and 30 °C (Chawla *et al*., 2009).

Zahan and co-workers (2015b) studied the effect of different incubation temperatures on the growth of *G. xylinus* and BC production. Different incubation temperatures were investigated (5°C, 20°C, 25°C, 27°C, 28°C, 30°C, 35°C and 40°C), and their reports show that there was no bacterial growth and BC production at 5°C and at 40°C, due to obvious harsh conditions while they suggested that 28°C is the most favorable temperature for growth of *G. xylinus* and BC production.

Another important factor that affects BC production is dissolved oxygen. Substrates have to be transported by diffusion in static cultures and due to the general availability of carbon sources, availability of oxygen might be a limiting factor for cellular metabolism and this could have a negative effect on production and quality of cellulose (Chawla *et al*., 2009).

BC production under oxygen-enriched air at different fructose concentrations was studied using a 50l internal loop airlift reactor. It was reported that supply of oxygen-enriched air instead of air resulted in an increase of 0.093 g/l/hr in BC production rate and BC yield was enhanced from 11% to 18%. Enhanced BC production was detected with decrease in both CO_2 evolution and concentration of other unknown substances, this suggested the efficient utilization of energy for synthesis of BC even with oxygen limitation (Chao *et al*., 2001).

3.7 Applications of BC

Due to its physico chemical characteristics and high purity, bacterial cellulose has offered a diverse range of application medically and industrially.

3.7.1 BC in Wound Healing

Biocompatibility is a major requirement for any biomedical material. This is the ability of the material to remain with a living tissue and not cause any allergic or toxic side effects (Czaja, *et al*., 2007). Bacterial cellulose fibres are thinner than that of plant cellulose; this feature makes it a highly porous material. This facilitates antibiotics and other medicines' transfer to the wound site as well as physically protecting against external infections (Chawla *et al*., 2009).

Wound healing is a dynamic process that involves the interaction of different cell types, extracellular matrix molecules and soluble compounds. The process of normal wound healing is a systematic process that involves homeostasis, inflammation, granulation tissue formation and remodelling but due to failure of some of these systematic processes, chronic wounds such as ulcers do not heal (Czaja *et al*., 2007).

Different types of wound dressings have been developed and used for the elimination of the hostile environment within the chronic wound and to promote proper healing. Dressings such as hydrogels, hydrocolloids, synthetic and biological membranes and alginate have been used for treating ulcers. Most effective wound dressings have been developed and designed to maintain a moist environment in the affected area since it was discovered that re-epithelization rate was increased if the wound was kept moist (Czaja *et al*., 2007)

A dry-fabricated bio-film (DFBF) made up of homogenized BC oxidized by hydrogen peroxide, alginate and chitosan was investigated for its suitability for wound dressings. It was reported that the mechanical strength and hydrophilicity of DFBF was increased by homogenized BC oxidized by H_2O2, the modified DFBF also exhibited desirable mechanical properties and hydrophilicity, water vapor transmission rate, biocompatibility, and the ability to control the release of anti-inflammatory substances. This study suggests the modified

DFBF a potential candidate for use in wound dressing due to properties it exhibited (Chang and Chen, 2016).

3.7.2 BC in Food Application

BC is a type of dietary fibre and is generally recognised as safe, BC as a dietary fibre has more advantages compared to other dietary fibre sources. These include: high purity, without a requirement for harsh processing conditions, bacteria can be grown in media such as fruit syrup, with the produced BC adapting the colour and flavour of the medium (Shih *et al*., 2014).

Historically in the Philippines, BC gels have been used as a food (Nata) and various flavours such as nata-de-pina and nata-de-coco have been incorporated based on the medium source (Ul-Islam *et al*., 2015). Nata has also been used to enhance the physicochemical, texture and quality of Chinese-style meatballs. Reports show that addition of 10% nata to Chinese-style meatballs resulted in products with acceptable textural qualities, thus, nata was suggested as a potential functional ingredient in Chinese-style emulsified meat products, which would be a good replacement for fat (Lin and Lin, 2004).

3.7.3 BC as a Cryoprotectant

Due to the high-water absorption capacity and high mechanical strength in the wet state, high crystallinity and the ultrafine network of BC, researchers have tried to immobilize various bacteria on BC for the purpose of alcoholic fermentations and industrial waste water treatments. The use of this food grade support for cell immobilization and as a cryoprotectant has unique advantages which include low toxicity, edibility, availability and cost effectiveness (Jagannath *et al*., 2010).

The effect of using BC as a carrier support and as a cryoprotectant for probiotic lactic acid bacteria has been studied and compared with other known cryoprotectants such as skimmed milk powder, sucrose as well as alginate microencapsulation. BC could have proven to be a physical barrier and overcome the detrimental effect of freezing and drying due to its fibrous structure, a 2 Log reduction was observed in BC sample sheets for the 60 days' period. The other cryoprotectants were more effective; however, BC provides extra dietary fibre (Jagannath *et al*., 2010).

Using BC as a cryoprotectant and an inert support for probiotic bacteria is a novel concept that offers an easy method for preserving bacteria over a short period of time (Jagannath *et al*., 2010). Other applications of BC are summarised in table 3.2.

Table 3.2: Application of BC and its derivatives

Field	Application	Chemical/Biochemical Functions
Food	Cryoprotectant	The cryoprotective and carrier support potential of BC was studied on lactic acid bacteria and it was reported that BC provides an easy method for the preservation of lactic acid bacteria for a short period of time (Jagannath *et al*., 2010).
	Inhibition of bacteria growth on processed meats	BC-nisin film has been reported to be effective in the reduction of total aerobic bacteria and control of *L. monocytogenes* on the surface of the frankfurters. BC-Nisin film appears to be a promising method to improve safety and prolonging the self -life of processed meats (Nguyen *et al*., 2008).
	Vegetarian food stuff	A new foodstuff called *Monascus*-nata was developed by fermenting BC with *Monascus purpureus* (normally used for colouration and flavouring of food and beverages). *Monascus*-nata complex had a better resistance to washing, heating, freezing, acidification and alkalization. The complex has a potential to be a novel foodstuff as vegetarian meat (Sheu *et al*., 2000)
Medicine	Artificial Skin	A biosynthesised membrane made up of pure BC called biofill was developed for the treatment of different categories of burns in 36 children. Biofill was reported to be more effective than the conventional method as it allowed the children live a life closer to normal and reduction in infection during treatment was recorded. There was also reduction in hospital cost which makes the use of biofill economical (Capelo and Alves, 1994).
	Nanocomposite	BC was used in combination with polyvinyl alcohol (PVA) to develop biocompatible nanocomposites (BC-PVA). It was reported that the developed composites had mechanical properties similar to that of cardiovascular tissues such as the aorta and heart valve leaflets. BC-PVA composite appears to be a potential material for the replacement of cardiovascular soft tissues (Millon and Wan, 2005).
	Wound dressing	Purified gelatinous membrane of BC was developed in Brazil and used as an artificial wound dressing. BC was reported to be more superior to conventional gauze due to its high mechanical strength in the wet state, low skin irritation and substantial permeability for liquids and gases (Yoshinaga, *et al*., 1997).
	Drug delivery	The drug release property of BC was investigated by coating generic paracetamol tablets with 1% w/v BC. Drug release from non-coated paracetamol tablets were faster compared with BC coated tablets. It was suggested that BC can be used as an aqueous film coating agent with reduced cost and better film forming properties (Amin *et al*., 2012).

Table 3.2 Continued

Field	Application	Chemical/Biochemical Functions
Fashion	Cloth production	A symbiotic mix of bacteria, yeast and other microorganisms called the Kombucha recipe was used for the production of BC on large scale level (≈30l). Clothes made from dried BC cannot be used for day to day wears due to its hydrophilicity but can be used as performance piece (Lee, 2011).
Others	Enzyme immobilization	Glucoamylase was immobilized on BC beads produced in an agitated medium. BC beads were reported to be promising for the preparation of immobilized glucoamylase for industrial applications (Wu and Lia, 2008).
	Tissue engineering	BC was implanted in mice subcutaneously in an *in vivo* biocompatibility study. Results showed BC integrated very well into the host tissue with cells infiltrating the BC network and no evidence of chronic inflammatory reaction or capsule formation was recorded (Andrade *et al*.,2010)
	Antimicrobial activity	BC on its own does not have any antimicrobial activity for the prevention of wound infection, but, when BC was loaded with silver nitrate ($AgNO_3$) and silver zeolite (AgZ), the results showed that both $AgNO_3$ and AgZ loaded BC hydrogels possess antimicrobial activities against *S. aureus* and *P. aeruginosa* (Gupta *et al*., 2016).
	Dental implants	BC Gengiflex membranes have proven to be a good alternative for guided tissue regeneration when used either alone or in relation with osteointegrated implants for periodontal disease treatment, dental implant and guided bone regeneration (Andrade *et al*., 2010).
	Production of Acoustic Transducer Diaphragms (ACDs)	Because of the high Young's modulus of BC, it has been reported to be an ideal material for ACDs. BC has been used for different types of headsets and high fidelity loud speaker units marketed by SONY CORPS (Iguchi *et al*., 2000 and Yoshinaga *et al*., 1997).

3.8 Aims of this Research

The aims of this research are:

1. Investigate the production of γ-PGA in shake flasks using 2 media and 2 bacilli strains
2. Characterise γ-PGA produced by the two strains, comparing the yield, molecular weight and using different analytical techniques to compare the γ-PGA
3. Scale-up of production of γ-PGA using a 5l fermenter
4. Investigate the production of BC using *G. xylinus* ATCC 23770 statically
5. Characterise produced BC in terms of wet weight, dry weight and using different analytical techniques for characterization
6. Investigate the potential of wet BC to act as a carrier support during freeze drying of 2 bifidobacteria strains
7. Milling of the produced BC sheets into powdered BC (PBC)
8. Investigate the inhibitory properties of 6 bifidobacteria against known pathogens
9. Investigate the cryoprotective effect of γ-PGA and PBC coatings on bifidobacteria and compare with known cryoprotectants
10. Investigate the effect of γ-PGA, PBC and a novel γ-PGA+PBC coatings on viability of probiotic bacteria in simulated gastric juice, simulated intestinal juice and simulated intestinal juice with bile salts.

4.0 MATERIALS AND METHODS

4.1 γ-PGA Production

4.1.1 Bacterial Strains

B. subtilis natto ATCC 15245 and *B. licheniformis* 9945a that have been screened were collected from the microorganism collection centre in the Microbiology laboratory of the University of Wolverhampton, Wolverhampton, UK. All stock cultures were freeze dried and stored at -20°C for preservation of bacterial strains. Frozen cells were resuscitated in TSB prior to use and grown on general purpose agar at 37°C for 24 - 48 hours.

4.1.2 Growth Media

Tryptone soya agar (TSA) and tryptone soya broth (TSB) were prepared according to the manufacturer's instructions (LAB M, UK). One-quarter strength Ringer's solution was prepared by dissolving one Ringer tablet (Oxoid, UK) in 500ml of deionised water and sterilised by autoclaving (Priorclave, UK) at 121°C for 15 minutes. Composition of TSB and TSA are shown in table 4.1.

Table 4.1: Composition of TSA and TSB

Composition of TSB			**Composition of TSA**		
Chemical	**Amount (g/l)**	**Company**	**Chemical**	**Amount (g/l)**	**Company**
Soy Peptone	5	Lab M, UK	Soy Peptone	5	Lab M, UK
Tryptone (Casein digest)	17	Lab M, UK	Tryptone (Casein digest)	15	Lab M, UK
NaCl	5	Lab M, UK	K_2HPO_4	2.5	Lab M, UK
K_2HPO_4	2.5	Lab M, UK	D-Glucose	2.5	Lab M, UK
D-Glucose	2.5	Lab M, UK	Agar number 2	12	Lab M, UK

GS medium and medium E were used to produce γ-PGA. Table 4.2 and table 4.3 show the contents of these media.

Table 4.2: Composition of GS Medium

Chemical	Quantity (g/l)	Company
L-glutamic acid	20	Sigma- Aldrich (UK)
Sucrose	50	Fisher Scientific (UK)
Potassium dihydrogen orthophosphate	2.7	Fisher Scientific (UK)
Di-sodium hydrogen phosphate anhydrous	4.2	Sigma-Aldrich(UK)
Sodium Chloride	50	Sigma-Aldrich (UK)
Magnesium sulphate heptahydrate	5	Sigma-Aldrich (UK)
Murashige-Skoog Vitamin solution	1ml/l	Sigma- Aldrich (UK)

Final pH of GS medium was aseptically adjusted to 6.8 using 3M NaOH and 3M HCl

All chemicals except sucrose and Murashige-Skoog solution were dissolved in deionised water and mixed on the magnetic stirrer (Heating Magnetic Stirrer Fisher Scientific Fb 15001, UK) for 4 hours at 200°C. The pH was adjusted to 4 and sterilised by autoclaving at 121°C at 1.035 bar for 15 minutes. Sucrose was dissolved in deionised water and autoclaved at 110°C and 0.35 bar for 30 minutes, this was done to prevent caramelization.

Sucrose was added aseptically to the remaining GS medium mixture and pH was adjusted aseptically to 6.8. 1ml/l Murashige-Skoog Vitamin solution (2.0 mg/ml glycine, 100 mg/ml myo-inositol, 0.50 mg/ml nicotinic acid, 0.50 mg/ml pyridoxine hydrochloride, 0.10 mg/ml thiamine hydrochloride) was filter sterilised using 0.45µm filter and added to the GS medium.

All chemicals used for medium E were dissolved in deionised water and left on the magnetic stirrer (Heating Magnetic Stirrer Fisher Scientific Fb 15001, UK) at 200°C for 4 hours. The pH was adjusted to 7.2 with 3M NaOH and 3M HCl. The medium was sterilised by autoclaving at 121°C at 1.035 bar for 15 minutes.

Table 4.3: Composition of Medium E

Chemical	Quantity (g/l)	Company
L- Glutamic acid	20	Sigma-Aldrich (UK)
Citric acid	12	Sigma-Aldrich (UK)
Glycerol	80	Avocado (UK)
Ammonium chloride	7	Sigma-Aldrich (UK)
Magnesium sulphate heptahydrate	0.5	Sigma-Aldrich (UK)
Iron(III)chloride hexahydrate	0.2	Sigma-Aldrich (UK)
Di-Potassium hydrogen orthophosphate anhydrous	0.5	Sigma-Aldrich (UK)
Calcium chloride dehydrate	0.15g/l	Fisher Chemicals (UK)
Manganese (II) sulphate monohydrate	0.2g/l	Sigma Aldrich (UK)

Final pH of medium E was adjusted to 7.2 with 3M NaOH and 3M HCl

4.1.3 γ-PGA Production in Shake Flasks Cultures

The production of γ-PGA in shake flasks from the *Bacillus* strains was carried out in triplicates. To prepare the seed culture, highly mucoid colonies were collected using a sterile inoculation loop and transferred to flasks containing 250ml sterile TSB. This was incubated aerobically in a rotary shaker (Innova 43, USA) at 37°C for 24 hours and 150 rpm.

At the end of the incubation period, 5% inoculum level was transferred into 500ml flasks containing 250ml of the production medium (GS medium or medium E). The flasks were corked with foam bungs and wrapped with aluminium foil. All flasks were incubated aerobically in a rotary shaker (Innova 43, USA) at 37°C for 96 hours as γ-PGA concentration has been reported to be at its maximum in *Bacillus* species at the late stationary phase of the culture medium (Bajaj and Singhal, 2011).

For the determination of pH and cell growth, samples were taken aseptically from the flask cultures at 0, 24, 48, 72 and 96 hours respectively.

4.1.4 Scale-Up of γ-PGA Production in Fermentation Culture

Batch fermentation was carried out using a 5l Electrolab fermenter (UK) to produce γ-PGA. The seed culture was prepared as with shake flasks experiment. The fermenter vessel was thoroughly washed and dried. GS medium without sucrose was then transferred into the fermenter. The pH probe was calibrated according to manufacturer's instructions, then the vessel was sealed properly and autoclaved at 121°C at 1.035 bar for 15 minutes. The Electrolab software was used to monitor the temperature, dissolved oxygen, pH and agitation; this was set up before the addition of the seed culture

Sucrose was sterilised by autoclaving at 110°C and 0.35 bar for 30 minutes and added aseptically to the sterile GS medium in the fermenter. Acid, base and antifoam bottles were carefully connected to the fermenter to avoid contamination. The Electrolab automatic pH control system was used to adjust the pH of the medium to 6.8 using 3M NaOH and 3M HCl. Dissolved oxygen probe was calibrated according to manufacturer's instructions and temperature maintained at 37° with the heat jacket.

5% of the seed culture was added to the GS medium in the vessel, stirring speed and airflow rate were set at 250rpm and 2.0 l/min respectively. Samples were collected periodically at 0, 24, 48, 72 and 96 hours respectively for determination of bacterial growth.

4.1.5 Bacterial Growth Determination.

Cell growth determination is important, as it will help to ascertain that the culture has not been contaminated and to compare polymer yield in different strains and media. Bacterial growth was determined using the Miles and Misra technique to calculate colony forming units/ml and this was done in triplicates. 4.5ml of Ringer's solution was transferred into test tubes and autoclaved at 121°C for 15 minutes and 1.035 bar. At the designated time, 5ml

samples were removed aseptically from the culture medium. 500 µl of each of the sample was transferred into one test tube containing sterile Ringer's solution and dilutions were performed serially from 10^{-1} to 10^{8}. 20µl of each dilution was transferred onto plates containing TSA, plates were incubated overnight at 37°C. Colony counts of the plates were taken; the average for each dilution was determined and the results were expressed as CFU/ml using the formula:

CFU/ml= average number of colonies x 50 x 1/dilution factor

The values obtained for the colony forming units/ml (CFU/ml) for all replicates (n=3) were converted to Log_{10} values, the values were statistically analysed and used to develop a fermentation growth curve.

4.1.6 Extraction of γ-PGA

To separate the cells from the γ-PGA containing broth, cell culture broth from the shake flasks or fermenter were collected after 96 hours and transferred equally into six centrifuge tubes and centrifuged (Sigma Laboratory Centrifuge, UK) for 30 minutes at 1700*g* and at 4°C to separate the cells from the broth. The supernatants containing γ-PGA were collected. Biomass pellets were made inactive by soaking in trigene (Medichem International, UK) before discarding. Samples containing γ-PGA were transferred into 1000ml Schott bottles and 95% (v/v) ethanol was added (1:4v/v) to precipitate the γ-PGA. The content of the bottles was gently stirred for ≈2 minutes and left undisturbed in the cold room for 72 hours to allow precipitation of γ-PGA to take place.

After 72 hours, wet γ-PGA which formed the sediment from the ethanol was collected by gently sucking out the ethanol without disturbing the sediment. The residue containing ethanol and γ-PGA was mixed thoroughly and centrifuged for 30 minutes at 1700*g*.

Supernatants containing ethanol were discarded while the γ-PGA residue was re-dissolved in water using a magnetic stirrer (Progen Scientific Limited, UK).

Once wet γ-PGA was properly dissolved in water, treated dialysis membrane tubes (Spectrum Labs, UK) were used to remove impurities that are lower than 10,000 Da. This was done by filling a 5l plastic beaker with deionised water, the dialysis tube with one end sealed with a plastic clip was placed in the water to prevent it from drying out. Wet γ-PGA was transferred into the tube through the open end and then sealed using a plastic clip. This was allowed to remain for 24 hours with the water changed at 2, 4 and 16 hours in order to maintain the concentration gradient.

After the dialysis process, pure γ-PGA was then transferred into round bottom flasks and swirled over a mixture of dry ice and cold ethanol at -20°C to freeze the pure γ-PGA.

The samples were freeze-dried using the freeze dryer (CHRIST LCG LYO Chamber Guard, Germany) for 72 hours at -40°C and at 5 Mbar pressure to obtain dry γ-PGA. Dry γ-PGA samples were collected after freeze-drying and weighed to determine the yield (g/l). Dry samples were then kept in desiccator while awaiting further qualitative analysis and for the probiotic tests.

4.1.7 Chemical Analysis of γ-PGA

4.1.7.1 Identification of γ-PGA

Identification of the isolated polymer was necessary. This was carried out using Fourier Transform Infra-Red spectroscopy (FT-IR).

FT-IR is a technique that is non-destructive, quick and which does not require external calibration. It involves the passage of infrared radiation through the sample to be analysed, the sample either transmits or absorbs the radiation and the resulting spectrum represents the

molecular absorption and transmission. Each absorption peak on the spectrum stands for the frequencies of vibrations between the bonds of the atoms that make up the material. FT-IR spectra can be employed as a qualitative analysis tool for the identification and determination of quality of unknown samples.

A Genesis II with Durascope (Mattson Instruments, UK) was used to identify the produced polymer. The loading stage of the machine was cleaned with tissue sprayed with acetone; a background scan was carried out then the polymer sample was loaded onto the diamond attenuated total reflector and scanned.

The peaks obtained from the different samples of γ-PGA produced during this research were compared with the standard peaks of commercial samples of γ-PGA for peaks identification.

4.1.7.2 Molecular Weight

Molecular weight (M_w) of γ- PGA is an important factor in its application, hence the need for the determination of molecular weight of the produced γ-PGA. The molecular weight (M_w), molecular number (M_n) and dispersity index (DI) were determined by using conventional aqueous based gel permeation chromatography (GPC). DI is a measure of the wideness of distribution of molecular weight in a polymer sample and it can be determined using the equation:

$$DI = \frac{Mw}{Mn} \geq 1$$

The closer the DI is to unity, the more uniform the molecular weight of the polymer. Analytes are separated by GPC based on their size; the analytes are passed through porus beads packed in a column. Smaller analytes easily enter the pores easily and spend more time in the column, hence, increasing their retention time. However, larger analytes cannot enter the pores like the smaller analytes and hence a shorter retention time for larger analytes.

Molecular weight analysis of produced γ-PGA was done at Smithers Rapra at Shrewsbury, UK and a Viscotek Triple Detector Array TDA301 with PL aquagel-OH guard plus 2 * PL aquagel-OH MIXED-H was used for this analysis. 0.2M $NaNO_3$, 0.01M NaH_2PO_4 at pH 7 were used as eluent (since γ-PGA is insoluble in organic solvents) with a flow rate of 1.0 ml/min at 30°C and a Refractive Index (RI) detector with pressure and light scattering. Data collection and analysis was done using the Malvern/Viscotek 'Omnisec' software.

Samples were prepared by adding 10ml of eluent to about 20mg of γ-PGA samples and samples were left overnight to completely dissolve. Samples were then warmed up at 50° C for 30 minutes and after cooling, the solution was filtered through a 0.45µm polyvinylidene filter directly into auto sample vials. For the calibration of the GPC system, a range of sodium polyacrylate calibrants from Agilent/Polymer Laboratories was used.

4.2 Production of Bacterial Cellulose

4.2.1 Microorganism

Screened *Gluconacetobacter xylinus* ATCC 23770 was used to produce bacterial cellulose and this was obtained from the microorganism collection centre of the Microbiology laboratory of the University of Wolverhampton, Wolverhampton, UK. All stock cultures were freeze dried and stored at -20°C for preservation of bacterial strains. Frozen cells were resuscitated in HS medium prior to use and grown on HS agar at 37°C for 48-72 hours

4.2.2 Growth Media

Hestrin and Schramm (HS) medium, the modified HS (MHS) medium and HS agar were used to produce bacterial cellulose, the components of the media are in table 4.4. All media and agar were prepared using deionised water and autoclaved (Priorclave, UK) at 121°C for

15 minutes. One-quarter strength Ringer's solution was prepared according to manufacturer's instructions.

Table 4.4: Composition of HS medium, MHS medium (contains all chemicals with the exception of citric acid) and HS agar.

Chemical	Quantity (g/l)	Company
Glucose	20	Sigma Aldrich (UK)
Yeast extract	5	Lab M (UK)
Bacteriological Peptone	5	Lab M (UK)
Citric Acid	1.15	Sigma Aldrich (UK)
Disodium Phosphate	2.7	Sigma Aldrich (UK)
Agar number 2 (for HS agar)	15	Lab M (UK)

Final pH was adjusted to 6 using glacial acetic acid

All chemicals were dissolved in deionised water apart from the Agar number 2 (LabM, UK), which was used for the preparation of HS agar and mixed on a magnetic stirrer (Progen Scientific Limited, UK) until fully dissolved. pH was adjusted to 6 with glacial acetic acid and autoclaved at 121°C for 15 minutes

The seed culture was prepared by collecting highly mucoid colonies of *G. xylinus* ATCC 23770 and transferring them aseptically into 250ml conical flasks that contain 100ml sterile HS medium. This was cultivated aerobically in a rotary shaker (Innova 43, USA) at 30°C for 72 hours at 150 rpm.

After the incubation period, the seed culture was shaken vigorously to dislodge the cells from the formed pellicles and then 5% of the starter culture was transferred into sterile flasks containing sterile HS medium and the MHS medium (MHS medium contained all the components of HS medium excluding citric acid). This was cultivated statically in an incubator (Laboratory Thermal Equipment LTD, UK) at 30°C for 14 days.

4.2.3 Bacterial Growth Determination

It was important to monitor the growth of the bacteria and to ensure foreign organisms have not contaminated the culture medium. Bacterial growth determination was determined using the Miles and Misra technique as previously explained. 20µl of diluted samples were transferred onto plates containing HS agar and incubated for 48 hours at 30°C. Colony counts of the plates were taken and the average for each dilution determined, and the result expressed as Log_{10} CFU/ml.

4.2.4 Extraction and Purification

Pellicles observed on the surface of the media on day 14 were removed and washed in 1% NaOH at 80°C for 15 minutes; this was done to remove the bacterial cells and medium components from the pellicles. The pellicles were further treated by boiling in distilled water and rinsing with distilled water until a neutral pH was achieved. The wet weight of pellicles was determined and the pellicles were stored at -20°C overnight in preparation for freeze-drying. The pellicles were freeze dried for 48 hours using the freeze dryer (CHRIST LCG LYO Chamber Guard, Germany) at -40°C and at 5 Mbar pressure to obtain dry cellulose sheets. Samples were collected after 48 hours, weighed to obtain yield (g/l) and kept in desiccator for further analyses and probiotic application.

4.2.6 Milling of Freeze Dried Bacterial Cellulose

Milling of the freeze-dried BC samples was imperative as some of the characterization analyses required powdered samples and for the probiotic experiment.

A small sample size was sent to C. Gerhardt Limited, UK for trial milling. The variable speed rotor mill Pulverisette 14 (Fritsch, Germany) was used to mill the samples into powder particles using an 0.08mm sieve ring.

This machine crushes samples rapidly and efficiently. A combination of force, shear and friction between the rotor blades and sieve ring is used for this function with a built-in temperature sensor that automatically stops the mill if it gets too hot during the milling process. The collection pan and lid were then placed in the mill and the lid was locked firmly. The rotor speed was set at 20,000 rpm and the mill was started. This machine has a maximum feed size of 10mm, therefore it was imperative not to overload the machine, Dried BC samples (≈1g) were fed slowly into the funnel. The samples were sheared by the rotor blades and then forced through the sieve ring into the collection pan. Milled samples were then collected from the collection pan.

After milling the samples, it was important to investigate if milling the samples had any adverse effect on the structure and composition of BC. Samples were then analysed using SEM, FT-IR and XRD. After confirming that the milling did not have any detrimental effect on the BC samples, scale-up production of BC was carried out and samples (≈25g) were then sent back to the company for milling.

4.2.7 Characterization of Bacterial cellulose

4.2.7.1 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR was employed to study the conformational characteristics of BC obtained HS medium and MHS medium**.**

A Genesis II with Durascope (Mattson Instruments, UK) was used to analyse the produced BC in a transmittance mode at wavelength ranging from 4000 cm^{-1} to 400 cm^{-1}. The loading stage of the machine was cleaned with tissue sprayed with acetone; a background scan was carried out then a little BC sample just enough to cover the disc hole was loaded onto the diamond attenuated total reflector and scanned.

The peaks obtained from the different samples of BC produced during this research were compared with the standard peaks of commercial sample cellulose for peaks identification.

4.2.7.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) makes use of electrons to form an image. A focused beam of energy high energy electrons produced at the top of microscope is used to generate a variety of signals at the surface of solid specimens. The accelerated electrons then discharge their energy as a variety of signals produced by electron-sample interactions when the incident electrons are decelerated in the solid sample. These signals together with the secondary electrons produce SEM images.

The BC produced during this research were analysed using SEM (Zeiss Evo 50 fitted with an Oxford EDX, Zeiss, UK). SEM revealed the external morphology and the fibre network of the BC samples. A thin layer of freeze-dried BC sample was coated with gold using an ion sputter-coater in order to improve the conductivity of the BC sample and SEM image quality (emscope SC 500). The samples were viewed and the images were captured digitally.

4.2.7.3 X-Ray Diffraction (XRD)

This is an analytical technique used in the identification of crystalline phases by their diffraction patterns. Properties such as tensile strength, hardness, solubility, melting point and stiffness are usually influenced by the crystallinity of the polymer.

Polymers usually have the crystalline and amorphous phases arranged randomly. A structure where atoms are arranged in a regular repeating pattern is the crystalline phase while the pattern of the amorphous phase is irregular. The atoms that are arranged regularly that signify the crystalline phase in the sample reflect the beam; and are represented by sharp and narrow

diffraction peaks on the XRD spectrum while the random atoms that represent the amorphous phase are represented by very broad peaks.

XRD was used in this study for the characterization of the crystallinity index and degree of polymerization of the BC samples. This was carried out at the University of Wolverhampton using the Empyrean PANalytical diffractometer (Philips: PW1770, UK). Dried BC sheet was powdered before analysis. Sample was then placed in a sampling tray to smooth the surface and get rid of irregularities and data collected.

4.3 Probiotics

4.3.1 Bacterial Strains for Probiotics Studies

Six bifidobacteria strains *B. breve* NCIMB 8807, *B. longum* NCIMB 8809, *B. adolescentis* NCIMB 702229, *B. animalis* NCIMB 702716, *B. infantis* NCIMB 702255 and *B. bifidum* NCIMB 702715 were used for this study. Four pathogenic strains: two Gram positive (*Listeria innocua* NCTC 11288, *Staphylococcus aureus* NCIMB 6571) and two Gram negative (*Salmonella typhimurium* WLV 73 Cardiff Collection and *Escherichia coli* W1485-K12 W-T Cardiff Collection) were also used for this investigation.

B. breve NCIMB 8807, *B. longum* NCIMB 8809 and the four pathogenic strains were obtained from the microorganism collection centre in the Microbiology laboratory of the University of Wolverhampton, UK. Stock cultures were freeze dried and stored at -20°C for preservation of bacterial strains. Frozen cells were resuscitated in Bifido Selective Medium (BSM) for the probiotic strains and TSB (for the pathogenic strains) prior to use and grown on BSM agar and general-purpose agar at 37°C for 24-48 hours. The other probiotic strains were purchased from the National Collection of Industrial, Marine and Food Bacteria (NCIMB) culture collection. Stock culture was delivered in ampoules; they were revived by

aseptically breaking the ampoules and sterile growth medium added to the ampoules, this was then spread onto BSM agar plates and incubated anaerobically at 37°C for 48-72 hours in anaerobic jars using anaerobic atmosphere generation bags (Sigma Aldrich, UK).

4.3.2 Growth Media

Bifido Selective Medium (BSM) and trypticase-phytone-yeast extract (TPY) were used for the growth and enumeration of bifidobacteria. It is important to note that TPY can be used for enumeration of bifidobacteria; however, this is not selective for the bifidobacteria, as it will not hinder the growth of other bacteria. BSM on the other hand is a selective medium used for the selective isolation, identification and enumeration of bifidobacteria species. It contains selective salts that inhibit the growth of moulds, enterococci, lactobacilli, streptococci and other Gram-negative bacteria. It also contains an azo compound that can be reduced by bifidobacteria; this gives the colonies a purple-pink colour. Table 5 shows the components of TPY broth and TPY agar.

4.3.3 Sterilization of γ-PGA

Sterilizing the produced γ-PGA before using it for probiotic application was unavoidable. This was done so that the residual cell population in the γ-PGA does not affect the probiotic test results. The method of sterilization used for this study was adopted from Bhat *et al*., 2013 where γ-PGA was sterilised by autoclaving at 0.35 bar at 110°C for 30 minutes.

Table 4.5: Composition of TPY medium and TPY agar

Chemical	Quantity (g/L)	Company
Lag phase tryptone	10	Lab (UK)
Neutralised soya peptone	5	Oxoid (UK)
Glucose	5	Thornton & Ross (UK)
Tween 80	1ml/l	Acros Organic (USA)
Yeast extract	2.5	Lab M (UK)
Cystein hydrochloride	0.5	Sigma Aldrich (UK)
Dipotassium phosphate, K_2HPO_4	2	Sigma Aldrich (UK)
Magnesium chloride hexahydrate, $MgCl_2 \cdot 6H_2O$	0.5	May and Baker (UK)
Zinc sulphate heptahydrate, $ZnSO_4 \cdot 7H_2O$	0.25	Sigma Aldrich (UK)
Calcium chloride, $CaCl_2$	0.15	Sigma Aldrich (UK)
Ferric chloride, $FeCl_3$	Trace	Sigma Aldrich (UK)
Agar number 2 (for TPY Agar)	15	Lab M (UK)

Final pH of medium adjusted to 6.5 ± 0.1 using 3 M NaOH and 3 M HCl. TPY was sterilized at 110ºC and 0.35 bar for 30 mins to prevent glucose caramelization

4.3.4 Sterilization of Bacterial Cellulose

Wet BC samples were cut into cubes of approximately 5mm and sterilized by autoclaving at 0.35 bar at 110°C for 30 minutes. Powdered bacterial cellulose (PBC) was sterilised by autoclaving at 0.35 bar at 110°C for 30 minutes. Samples were analysed with FT-IR and SEM to check for any adverse effect on the structure and composition of the cellulose samples

4.3.4 Immobilization of *B. breve* NCIMB 8807 and *B. longum* NCIMB 8809

Wet cellulose sheets were cut into equal sized cubes (approximately 5mm) and autoclaved at 0.35 bar at 110°C for 30 minutes. Colonies of bifidobacteria were inoculated into 200ml sterile BSM broth and cultivated anaerobically at 37°C for (16 hours for *B. longum* NCIMB 8809 and 22 hours for *B. breve* NCIMB 8807). Cells were harvested by centrifugation at 1700*g* for 30 minutes and cell pellets washed using sterile phosphate buffer saline. 100ml

sterile BSM broth was added to the cell pellet, mixed using a vortex mixer (Stuart Scientific, UK) and allowed to stand for 15 minutes. 20ml of this mixture was added to sterile centrifuge tubes containing approximately 20g cut cubes of BC. The centrifuge tubes were then transferred into glass jars, an atmosphere generation system (Oxoid, Anaerogen, UK) was used to generate an anaerobic environment and then incubated using rotary shaker (Innova 43, USA) at 37°C for 48 hours.

Control samples were prepared by adding sterile cut cubes of BC to sterile BSM broth and inoculated under same conditions as other samples.

After 48 hours, the cubes of BC were removed aseptically, stored at -20°C overnight and then freeze dried at -40°C and 5 Mbar pressure (CHRIST LCG LYO Chamber Guard, Germany) for 48 hours. SEM was used to investigate attachment of cells to cellulose fibres.

4.3.5 Comparing the Effect of Known Cryoprotectants with PBC and γ-PGA on Probiotic Strains

The effect of skimmed milk powder (SMP) and sucrose were compared with using PBC and γ-PGA as cryoprotectants for *B. breve* NCIMB 8807, *B. longum* NCIMB 8809, *B. adolescentis* NCIMB 702229, *B. animalis* NCIMB 702716, *B. infantis* NCIMB 702255 and *B. bifidum* NCIMB 702715. Skimmed milk powder (SMP) (Sigma Aldrich, UK), sucrose (Sigma Aldrich, UK), PBC and γ-PGA were sterilised by autoclaving at 0.35 bar at 110°C for 30 minutes.

Bifidobacteria strains were inoculated in TPY broth overnight at 37°C anaerobically. Cultures were then centrifuged (Sigma Laboratory Centrifuge, UK) at 1700*g* for 30 minutes. The supernatant was discarded and cell pellet washed using sterile phosphate buffered saline (PBS). This was then centrifuged again and the PBS discarded.

Cells were then thoroughly mixed using the vortex mixer (Stuart Scientific, UK) with 10ml solution of sterile 5% SMP, 5% sucrose, 5% γ-PGA and 5% PBC; for the control sample, which had no cryoprotectant, 10ml sterile distilled water was added to the test tubes. To evaluate the number of viable cells before freeze drying, samples were taken from each group and serially diluted and plated on BSM agar plates and incubated anaerobically at 37°C for 48 hours. The samples were incubated at room temperature for 1 hour and stored in an ultra-low temperature freezer (U101 Innova, New Brunswick, UK) at -80°C overnight and freeze-dried (CHRIST LCG LYO Chamber Guard, Germany) at -40°C and 5 Mbar pressure for 48 hours.

After the freeze-drying process, 10 ml sterile PBS was used to rehydrate the samples and the number of viable cells present was determined. This was done by serial dilution and plating on BSM agar. Plates were incubated anaerobically at 37°C for 48 hours.

All tests were done in triplicates and carried out three times.

4.3.6 Antimicrobial Effect of Probiotic Strains on Food Pathogens.

4.3.6.1 Preparation of Cell-Free Culture Supernatants (CFCS)

Probiotic strains were grown in sterile TPY broth anaerobically at 37°C. The cell culture was centrifuged (Eppendorf centrifuge 5804R, Germany) at 13,000*g* for 10 minutes at 4°C, the pellet was discarded and the supernatant (CFCS) was transferred into sterile centrifuge tubes. It was important to confirm the absence of cells from the CFCS; hence an aliquot of the CFCS was taken for serial dilution, the pH of the supernatant was recorded and then two tubes were neutralised to pH 7 by the addition of 3M NaOH.

All the samples were filter-sterilised using a sterile 0.22µm-pore size micro filter.

4.3.6.2 Well Diffusion Assay

The well diffusion assay is an analysis that is usually carried out to investigate the potential mechanism involved in the inhibition of the growth of pathogenic bacteria. This was done to investigate if the probiotic strains had any inhibitory effect on the growth of pathogenic bacteria. Overnight cultures of the selected pathogens (*L. innocua* NCTC 11288, *S. aureus* NCIMB 6571, *S. typhimuirum* WLV 73 Cardiff Collection and *E. coli* W1485-K12 W-T) were lightly streaked on TSA plates using sterile cotton swab sticks and a sterile 6mm metal cylinder corer was used to make wells on the streaked TSA plates. 60µl of CFCS was transferred into each well. This was repeated for CFCS with pH adjusted to 7.0. Plates were incubated at 37°C for 24 - 48 hours and the antimicrobial activity was recorded as growth-free inhibition zones around the wells. The control was sterile TPY medium with pH 6.5.

The two groups of CFCS were then concentrated by storing in the freezer overnight and freeze dried, the freeze-dried samples were then reconstituted by adding 2mls of sterile distilled water and the antimicrobial activity was investigated using the method described above.

4.3.6.3 Organic Acid Production

Investigating the mechanism involved in the antibacterial activity of these strains was important and the reduction in pH. 250ml sterile TPY medium was inoculated with a 24-hour culture (1% v/v) of each of the probiotic strains (in separate flasks); the flasks were then incubated anaerobically at 37°C in a rotary shaker (Innova 43, USA). Samples were taken at 0, 8, 24, and 48 hours for organic acid assay, pH and for enumeration of bacterial growth. These samples were then centrifuged (Eppendorf centrifuge 5804 R, Germany) at 3200 rpm, 4°C for 30 minutes to obtain the supernatants which were collected in sterile centrifuge tubes

and filter sterilised using 0.22µm pore size micro filter and labelled accordingly for the organic acid assay.

Acetic acid and lactic acid are organic acids known to be released during the fermentation of probiotic strains in culture medium, hence the concentration of these two organic acids were determined at 0, 8, 24 and 48 hours.

For the organic acid analyses, the Megazyme assay kits (Acetic acid, D-Lactic and L-Lactic acid and Citric acid) were purchased from Megazyme (Bray, Ireland) and samples were prepared according to instructions. A spectrophotometer (WPA Biowave II Mets, USA) set at wavelength of 340nm and 1cm path cuvette was used to measure the absorbance; it was read against water to balance the spectrophotometer. For each organic acid analysed, the absorbance difference was determined (A_1-A_2) where A_1 is the initial reading before addition of different reagents and A_2 is the final reading except for Lactic acid analysis (A_2-A_1) where A_2 was greater than A_1. Once the absorbance difference has been determined, the absorbance difference of the blank (sample containing all reagents except the sample to be analysed) was then subtracted from the absorbance difference of the sample and $\Delta A_{(organic\ acid)}$ was obtained.

The concentration of the organic acid was then calculated using the formula:

$$C = V \times MW / \varepsilon \times d \times v \times \Delta A_{(organic\ acid)} \quad [g/l]$$

V= final volume (ml)

MW= molecular weight of organic acid (g/mol)

ε= extinction coefficient of NADH at 340nm = 6300 [1 x mol^{-1} x cm^{-1}]

d= light path [cm]

v= sample volume [ml]

4.3.7 Protection in Simulated Gastro Intestinal Fluids

B. longum NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 were selected for testing in simulated gastrointestinal fluids experiments. The protective effect of 2.5% and 5% (w/v) PBC, 2.5% and 5%(w/v) γ-PGA and a combination of γ-PGA and PBC [2.5% (w/v) PBC and 2.5% (w/v) γ-PGA] was investigated when cells were exposed to different gastrointestinal fluids: simulated gastric juice secreted in the stomach, simulated intestinal fluid secreted in the small intestine and a mixture of simulated intestinal juice and bile salts secreted in the small intestine.

The simulated gastrointestinal fluids tests were carried out in an Anaerobic Workstation (Electrotek, UK) where a microaerophilic environment was maintained. To confirm the quantity of the gases present in the cabinet after flushing the system with nitrogen, a gas analyser (Geotech GA 2000, UK) was placed in the cabinet, this showed the quantity of the different gases in the cabinet (9.9% CO_2, 4.4% O_2 and 85.3% N_2).

4.3.7.1 Protection in Simulated Gastric Juice

Heavy loss in the viability of probiotic strains are usually recorded when they pass through the stomach because of its acidity. The required time for food to pass through the stomach varies with different individuals and can be between 0.5-4.5 hours (Hsieh, *et al.*, 2009).

The protective effect of the biopolymers was tested on the viability of *B. longum* NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 when stored in simulated gastric juice for 4 hours. The components of simulated gastric juice are shown in table 4.6 (Bhat *et al*., 2015).

The solution was sterilised by passing it through 0.22µm syringe filter. The protocol for cryoprotection was followed for this test, viability of protected and unprotected cells was measured hourly for 4 hours.

Table 4.6: Composition of Simulated Gastric Juice

Chemical	Quantity (g/l)	Company
Pepsin Powder	3.2	Sigma Aldrich (UK)
Sodium chloride	2	Sigma Aldrich (UK)
1M Hydrochloric acid	20ml/l	Sigma Aldrich (UK)

Made up to 900ml using deionised water; mixed together, pH adjusted to 2 using 1M HCl or 1M NaOH and topped up to 1000ml.

4.3.7.2 Protection in Simulated Intestinal Juice (SIJ)/Bile Salts

It is important to investigate what happens to the probiotic strains after passing through the stomach to the small intestine. Viability in simulated intestinal juice and bile salts were carried out using the same protocol as simulated gastric juice. The components of simulated intestinal juice are shown in table 4.7 (Ikawo, 2014).

Table 4.7: Composition of Simulated Intestinal Juice (SIJ) and Simulated Intestinal Juice with Bile Salts (SIJ+B)

Chemical	Quantity (g/l)	Company
Monobasic potassium phosphate	6.8	Sigma Aldrich (UK)
Pancreatine	1	Sigma Aldrich (UK)
0.2N Sodium hydroxide	77ml/l	Sigma Aldrich (UK)
Bile Salts	1%	Sigma Aldrich (UK)

Dissolved in deionised water; final pH adjusted to 6 using 1M NaOH or 1M HCl. It was filter sterilised by passing through a 0.22µm syringe filter.

Since bile salts are released into the small intestine during the digestion of fatty foods, 1% bile was added to the simulated intestinal juice and pH adjusted to 6 as well.

4.4 Statistical Analyses

All statistical analyses were carried out using Microsoft Excel 2016 and GraphPad Prism 7. One-way ANOVA using the Tukey multiple comparison test for non-parametric analysis to determine the difference between individual groups in a data set. A comparison is statistically significant if the P value is ≤ 0.05.

5.0 RESULTS - BACTERIAL PRODUCTION OF POLY-GAMMA-GLUTAMIC ACID

5.1 γ-PGA Production in Shake Flasks

5.1.1 Assessment of Growth of Different Strains in Different Media

B. subtilis natto ATCC 15245 and *B. licheniformis* ATCC 9945a were grown in GS medium and medium E at 37°C and 150 rpm for 96 hours with 5% seed culture in shake flasks with pH adjusted to 6.8-7.2. Samples were taken at regular intervals (0, 24, 48, 72 and 96 hours) to determine the pH of each medium and the bacterial growth. Determination of cell growth was important to be sure the fermentation broth is devoid of contaminants and to determine the appropriate medium for the different strains due to the difference in composition of the media. The Miles and Misra plating technique was used for the cell growth determination of all bacterial strains in GS medium and medium E. Cell colonies observed on the plates after incubating overnight at 30°C were counted, the Logarithm (Log_{10}) of the number of colony forming units (CFU/ml) was derived and this was used for the construction of growth curves (figures 5.1 and 5.2)

All experiments were carried out in triplicates and statistically analysed.

5.1.2 *B. subtilis* Natto ATCC 15245 in GS Medium and Medium E

When *B. subtilis* natto ATCC 15245 was grown in GS medium, an increase in cell growth was observed in the first 24 hours of incubation from 7.23 Log CFU/ml - 8.60 Log CFU/ml Maximum mean cell population of 8.60 Log CFU/ml was reached within the first 24 hours of incubation. This was followed by a decline in cell count between 24 and 48 hours as seen in figure 5.1. A decline in cell growth was observed between 48 hours and 72 hours from 8.31 Log CFU/ml - 7.67 Log CFU/ml. This was followed by a further decline in cell number at 96hours from 7.67 Log CFU/ml - 7.44 Log CFU/ml.

In medium E, maximum mean cell population of 8.55 Log CFU/ml was only achieved at 72 hours unlike GS medium. Growth of cells in medium E was a gradual one as against GS medium where maximum mean cell population was achieved at 24 hours. After 72 hours, a reduction in cell growth was observed but this was not as rapid as the decline in cell growth observed in GS medium.

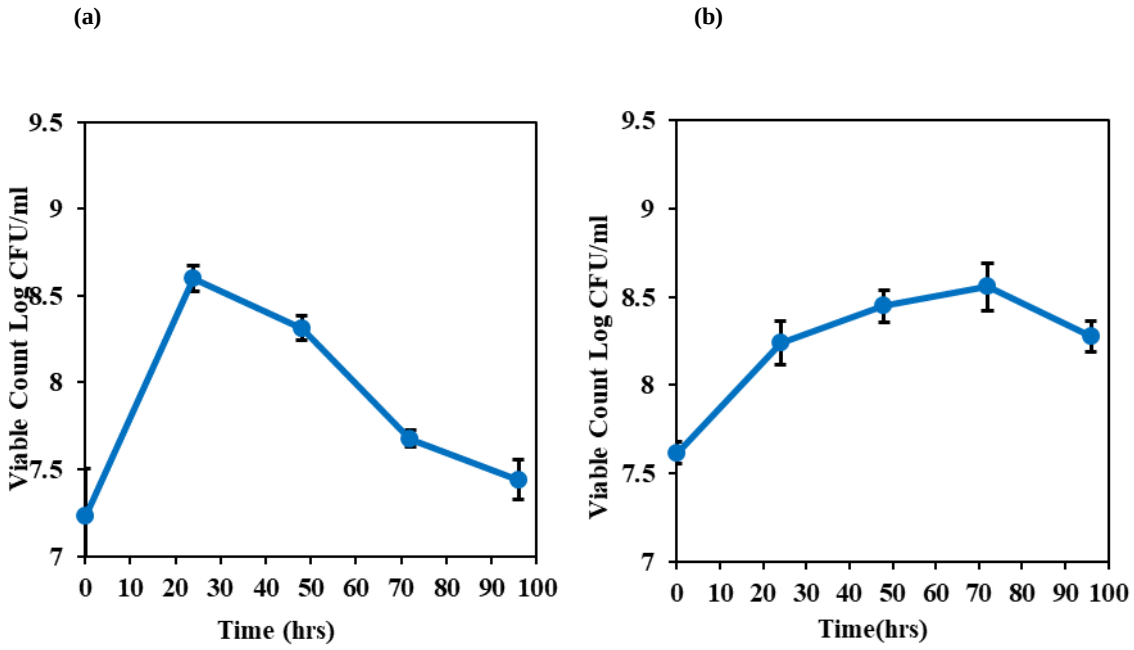


Figure 5.1: Growth curves (mean ±SE) for *B. subtilis* natto ATCC 15245 when grown in (a) GS medium, and (b) medium E at 150 rpm and 37°C for 96 hours to evaluate the production of γ-PGA (n=3)

5.1.3 *B. licheniformis* ATCC 9945a in GS Medium and Medium E

When *B. licheniformis* ATCC 9945a was grown in GS medium, an increase in cell growth was observed in the first 24 hours (figure 5.2a). Maximum cell population of 8.75 Log CFU/ml was achieved at this stage. This was followed by a stationary phase of 8.59 Log CFU/ml at 48 hours to 8.64 Log CFU/ml at 72 hours, followed by a decline phase with a reduction in cell growth from 8.64 Log CFU/ml at 72 hours to 8.07 Log CFU/ml at 96 hours.

When *B. licheniformis* ATCC 9945a was grown in medium E, maximum cell population of 9.07 Log CFU/ml was achieved in the first 24 hours (figure 5.2b) like when grown in GS medium, this was followed by a decline in cell growth at 48 hours, followed by a slight increase at 72 hours. A reduction in cell growth was observed after 72 hours, this is similar to the pattern observed when *B. subtilis* natto ATCC 15245 was grown in medium E where cell death was gradual and not rapid.

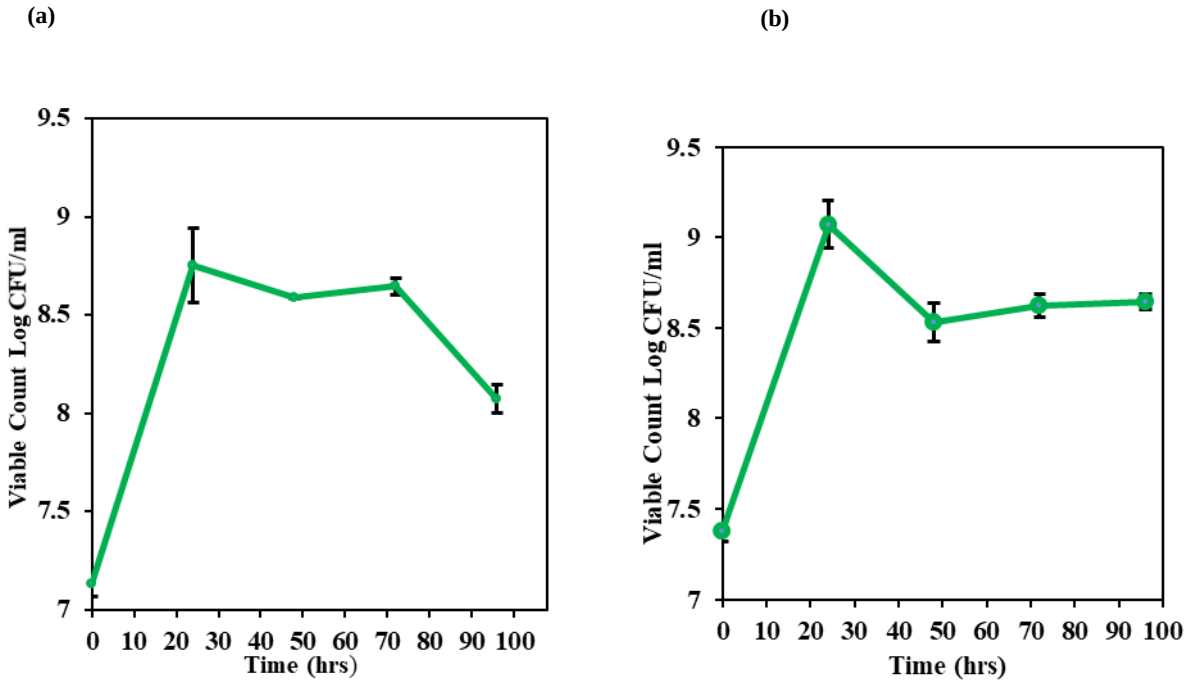


Figure 5.2: Growth curves (mean ±SE) for *B. licheniformis* ATCC 9945a when grown in GS medium (a), and medium E (b) at 150 rpm and 37°C for 96 hours to evaluate the production of γ-PGA (n=3)

5.2 Change in pH with Time

During the production of γ-PGA in GS medium and medium E, samples were taken at intervals (time zero and after every 24 hours) to determine the pH of each medium.

From the results (table 5.1), after 24 hours, a decrease in pH was observed during production with GS medium from pH 6.80 - pH 6.16, this remained the same until after 48 hours when

an increase in pH was recorded from pH 6.16 - pH 6.20. A further increase was also recorded at 96 hours. In medium E, the initial pH of 7.2 decreased to 6.69 after 24 hours, reduction in pH was observed throughout the incubation period with a final pH of 6.27 at 96 hours.

Table 5.1: Change in pH with time when *B. subtilis* natto ATCC 15245 was grown in GS medium and medium E at 37°C, 150 rpm for 96 hours

Time (h)	GS Medium (pH)	Medium E (pH)
0	6.8	7.2
24	6.16	6.69
48	6.16	6.52
72	6.20	6.34
96	6.31	6.27

5.3 Polymer Yield

It is important to understand the difference in γ-PGA yield based on producing strain and the production medium. This would help in the selection of the best culture medium and bacterial strain for scale-up production of γ-PGA for probiotic application.

To investigate the yield and effect of different culture media on γ-PGA production by the bacterial strains used in this research, the strains were grown individually in GS medium and medium E. They were incubated aerobically for 96 hours at 37°C as past studies have reported that maximum production during batch fermentation was achieved after 96 hours of incubation (Shih *et al*., 2002).

Polymers produced after 96 hours of cultivation were purified and obtained as dry powders. They were then weighed to determine the yield in g/l. Figure 5.3 shows the average γ-PGA yield recorded for the different strains in the media investigated.

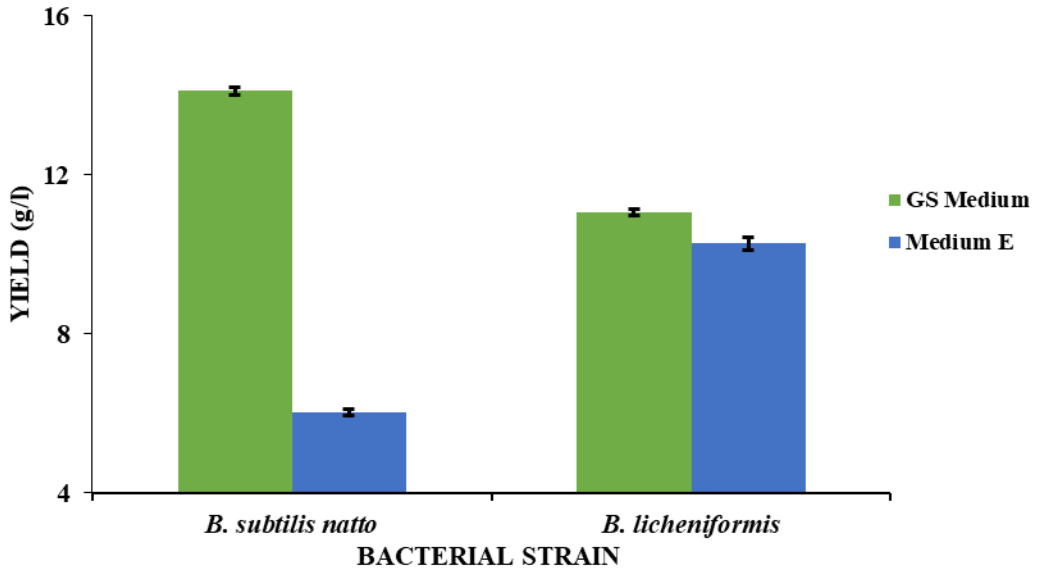


Figure 5.3: Yields (mean ± SE) of γ-PGA (g/L) produced by different *B. subtilis* natto ATCC 15245 and *B. licheniformis* ATCC 9945a in GS medium and medium E at 37°C for 96 hours. (n=3)

When *B. licheniformis* ATCC 9945a was used for γ-PGA production in GS medium and medium E, the average γ-PGA yield was similar in both media. An average yield of 11.03g/l was recorded in GS medium while an average yield of 10.27g/l was recorded in medium E. However, this was not the case when γ-PGA was produced from *B. subtilis* natto ATCC 15245 in GS medium and medium E. An average yield of 14.11g/l was recorded when *B. subtilis* natto ATCC 15245 was grown in GS medium while an average yield of 6.03g/l was recorded when *B. subtilis* natto ATCC 15245 was grown in medium E.

The highest yield of 14.11g/l was obtained when *B. subtilis* natto ATCC 15245 was grown in GS medium.

5.4 Identification of γ-PGA Using FT-IR Spectroscopy

It was important to confirm the identity of the obtained polymers. The polymer obtained when cells were grown in GS medium was whitish in colour while a brown coloured polymer was obtained when cells when cells were grown in medium E.

For identification, the obtained polymers were analysed using FT-IR spectroscopy and the resulting scans were compared with commercial samples of γ-PGA. The polymers obtained were identified as γ-PGA. Figures 5.4 and 5.5 show the absorption infra-red spectra of the obtained polymers as compared with commercial γ-PGA. Each spectrum is a mean of 3 spectra. The FT-IR absorption spectrum of commercial γ-PGA sample shows strong characteristic amide absorption peak at 1563cm^{-1}, strong hydroxyl absorption at 3292cm^{-1} which is due to bound hydroxyl group and adsorbed water molecules, strong carbonyl absorption peak is also noticeable at 1387cm^{-1} while the C-N group and N-H group were noticeable at 1074cm^{-1} and 527cm^{-1} respectively. The spectra obtained from isolated γ-PGA samples had similar peaks that are comparable with that of commercial γ-PGA sample (Figure 5.4 and 5.5).

5.5 Molecular Weight of γ-PGA

The molecular weight of γ-PGA influences its properties and applications; hence it is imperative to assess the molecular weight and dispersity index (DI) of the produced polymer. The dispersity index is an essential characteristic of γ-PGA, as the nearer the value of DI is to unity, the more the homogeneity of the γ-PGA is as regards its molecular weight.

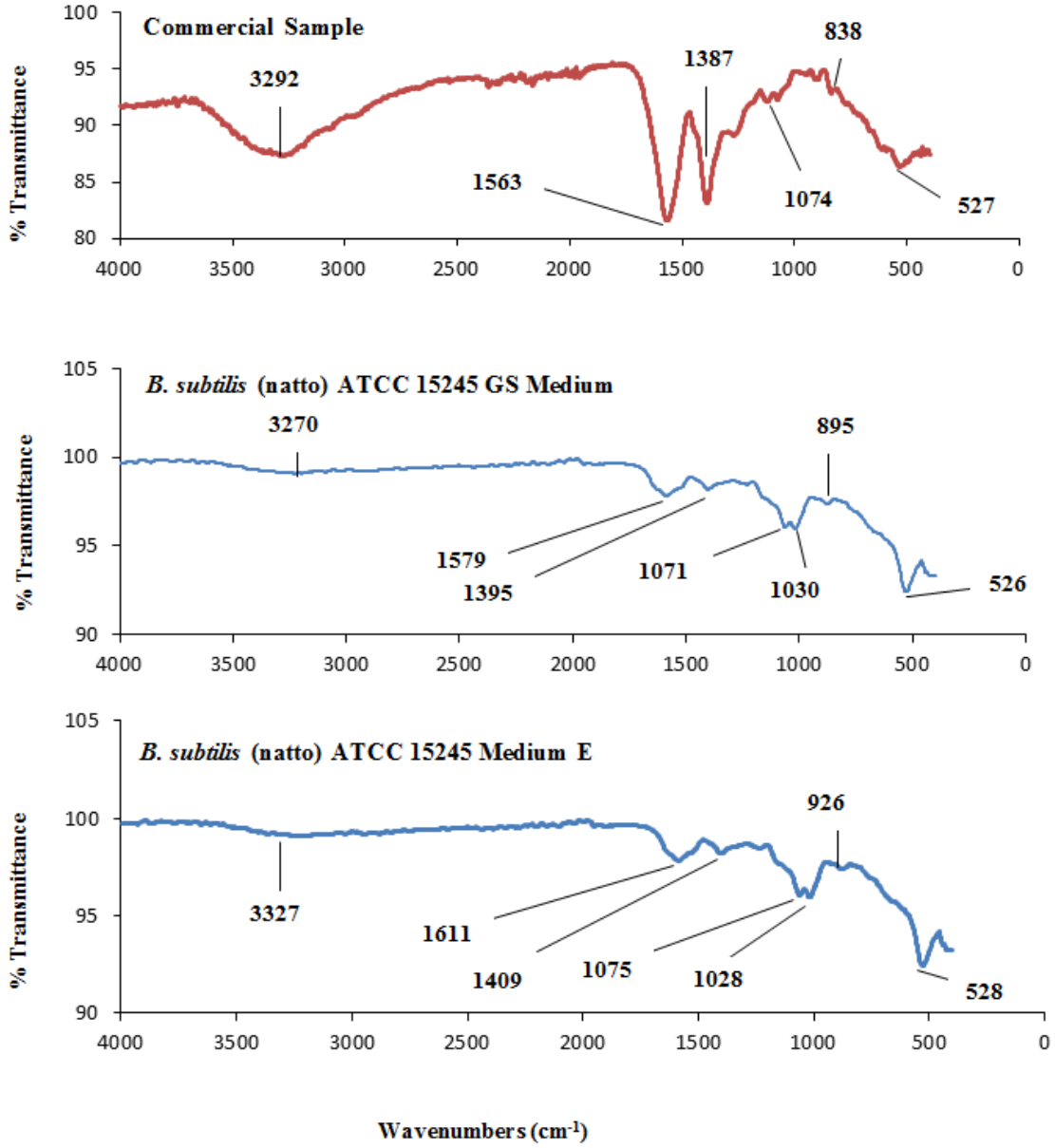


Figure 5.4: FT-IR spectra of γ-PGAs produced by *B. subtilis* (natto) ATCC 15245 in GS medium and medium E at 37°C and 150 rpm for 96 hours compared with the spectra of a commercial γ-PGA sample. Each spectrum is an average of 3 spectra (n=3)

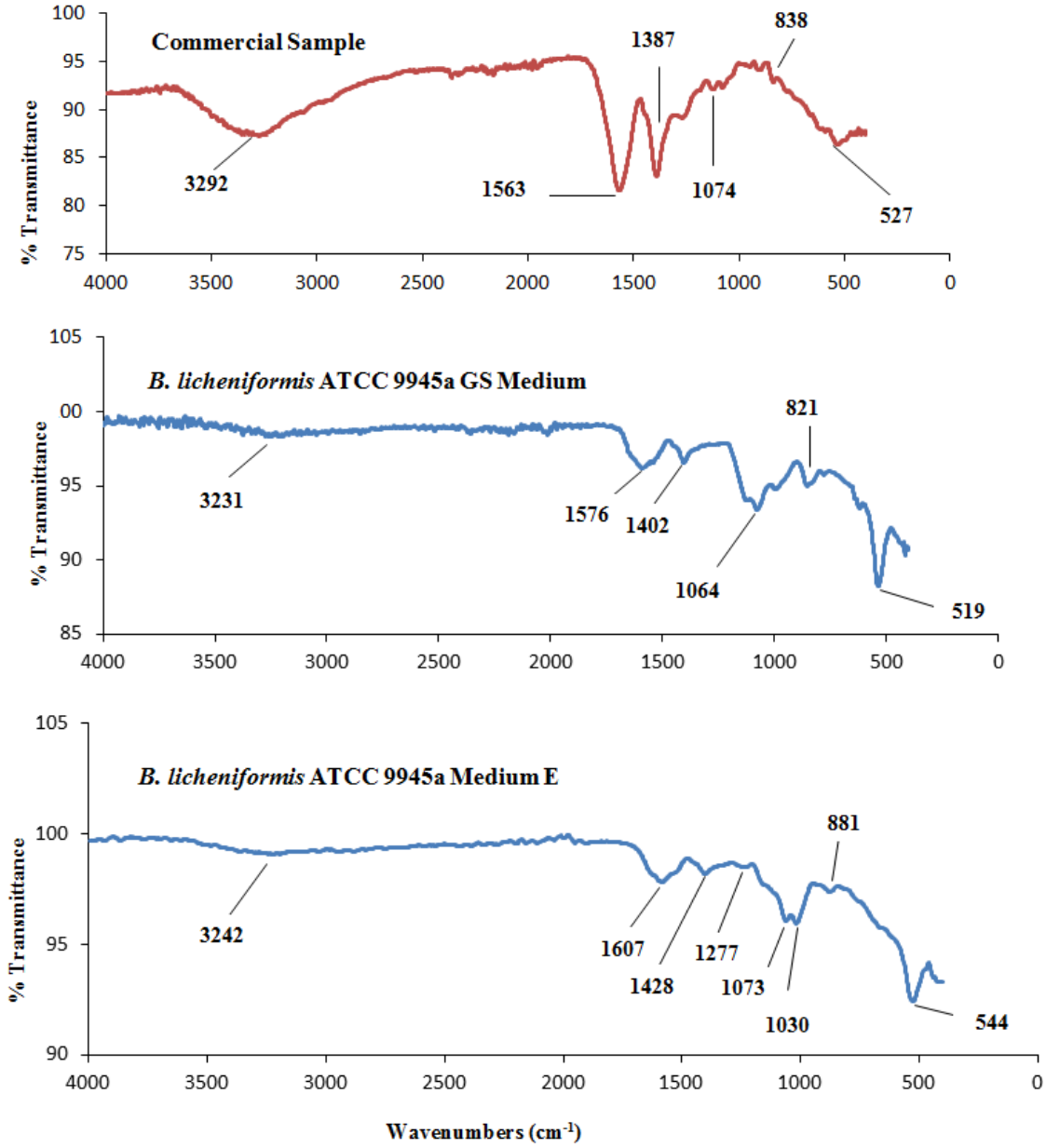


Figure 5.5: FT-IR spectra of γ-PGAs produced by *B. licheniformis* ATCC 9945a in GS medium and medium E at 37°C and 150 rpm for 96 hours compared with the spectra of a commercial γ-PGA sample. Each spectrum is an average of 3 spectra (n=3)

The molecular weight of γ-PGA of *Bacillus* species ranges from 10^5 to 10^6 and DI of between 2-5 usually recorded (Shih and Van, 2001). This is dependent on the source of its production (bacterial strain, culture conditions, media composition and action of enzymes excreted by microbes in the late stationary phase of growth) (Khalil *et al*., 2017).

γ-PGAs with higher molecular weight were obtained when *B. licheniformis* ATCC 9945a was used for γ-PGA production in both media with molecular weights of 1620 k Da in medium E and 1420 k Da in GS medium. Molecular weight of γ-PGAs obtained when *B. subtilis* natto ATCC 15245 (1280 k Da in GS medium and 1100 k Da in medium E) was used for production in both media were lower compared to that obtained from *B. licheniformis* ATCC 9945a. Table 5.2 shows the molecular weight and DI of γ-PGAs produced by *B. subtilis* natto ATCC 15245 and *B. licheniformis* ATCC 9945a in two media as analysed by aqueous based GPC.

Table 5.2: Molecular weight and dispersity of γ-PGA produced by (a) *B. subtilis natto* ATCC 15245 and (b) *B. licheniformis* ATCC 9945a in GS and E media at 37°C and 150 rpm for 96 hours. Analysis was carried out using GPC (PL aquagel-OH guard plus 2 * PL aquagel-OH MIXED-H)

(a) *B. subtilis* natto ATCC 15245

Medium	**Molecular Weight Da (M_w)**	**Molecular Number (M_n)**	**Dipersity (M_w/M_n)**
GS	1,280,000	535,000	2.4
E	1,100,000	520,000	2.1

(b) *B. licheniformis* ATCC 9945a

Medium	**Molecular Weight Da (M_w)**	**Molecular Number (M_n)**	**Dipersity (M_w/M_n)**
GS	1,420, 000	970,000	1.5
E	1,620,000	828,000	2.0

5.6 Scale-Up Production of γ-PGA by *B. subtilis* Natto ATCC 15245

After investigating the production of γ-PGA by *B. subtilis* natto ATCC 15245and *B. licheniformis* ATCC 9945a in GS medium and medium E in shake flasks and after characterization of the produced polymers, it was important to scale up production in a more controlled environment to increase polymer yield for the probiotic experiment. *B. subtilis* natto ATCC 15245 was chosen for γ-PGA production and probiotic application.

Growth of *B. subtilis* natto ATCC 15245 and γ-PGA production was studied using a 5l Electrolab fermenter (UK) where temperature, pH, agitation, aeration and dissolved oxygen were monitored and controlled.

The fermentation was run for 96 hours, at pH 6.80, temperature was maintained at 37°C and samples were taken at interval to measure viability.

During the fermentation in a 5l fermenter, *B. subtilis* natto ATCC 15245 reached its maximum cell count (8.57 Log CFU/ml) after 72 hours followed by a reduction in cell count to 8.16 Log CFU/ml after 96 hours (Figure 5.6). The γ-PGA yield by *B. subtilis* natto ATCC 15245 after extraction was 24.15±0.57 g/l.

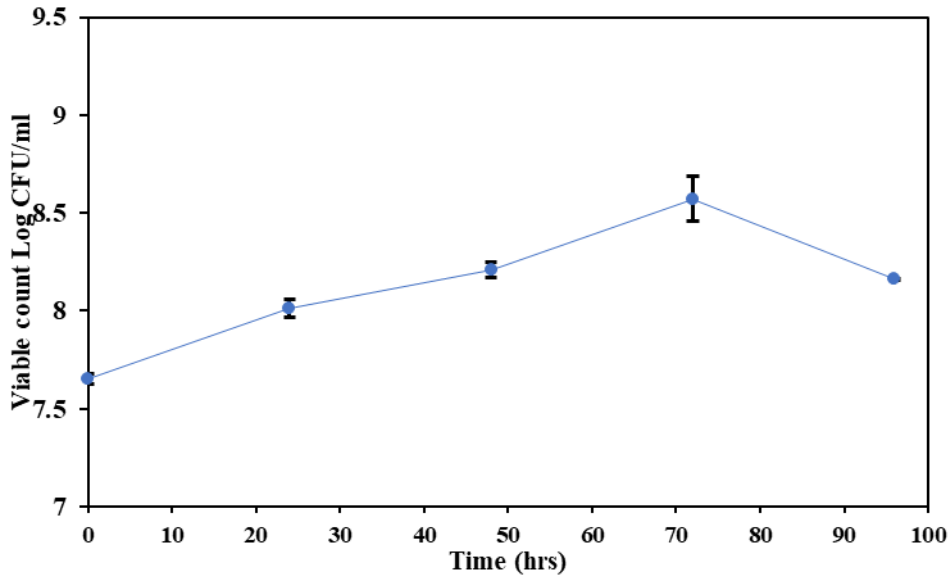


Fig 5.6: Growth of *B. subtilis* natto ATCC 15245 during fermentation in a 5l fermenter(mean ±SE). Cells were grown at 37°C for 96 hours in GS medium followed by γ-PGA recovery from the medium (n=2)

5.6.1 FT-IR

Purification and identification of the obtained polymer was very important, hence obtained polymer was purified by dialysis as described earlier (section 4.1.6) followed by lyophilisation and FT-IR was used for the identification of the obtained polymer

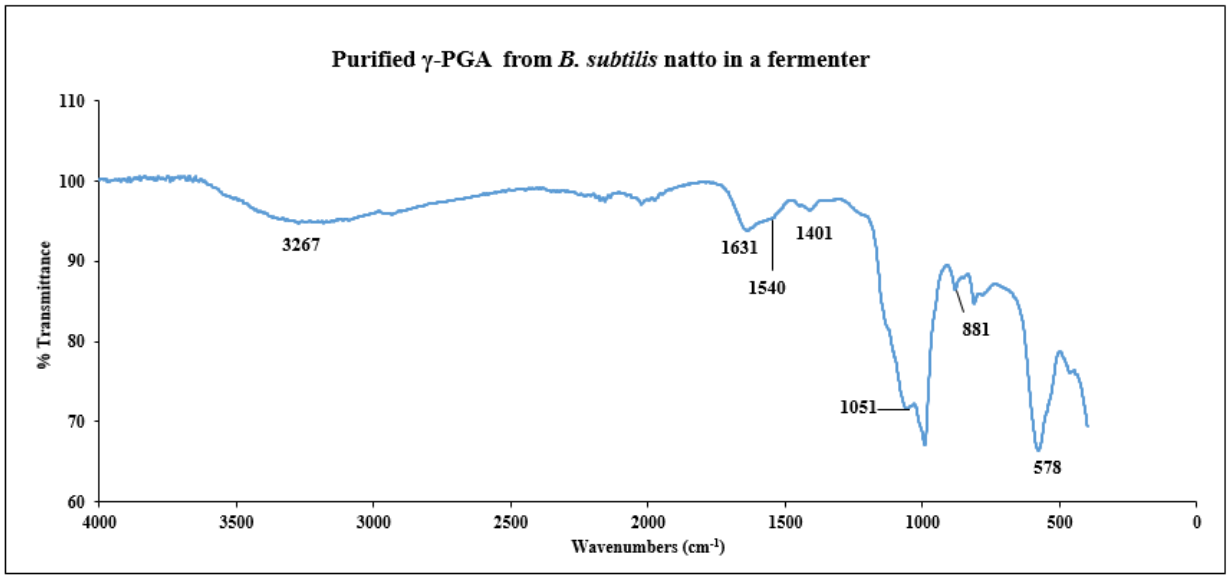


Fig. 5.7 FT-IR spectrum of purified γ-PGA obtained from fermentation of *B. subtilis* natto ATCC 15245 in GS medium in a 5l fermenter for 96 hours at 37°C

Peaks that were observed in the FT-IR spectrum (figure 5.7) were similar to those of commercial γ-PGA and γ-PGA obtained from the previous shake flasks experiments.

The peaks were observed at 3267cm^{-1},1631cm^{-1}, 1540 cm^{-1}, 1401cm^{-1}, 1051cm^{-1}, 881cm^{-1}, and 558cm^{-1} and these peaks correspond to the hydroxyl group, amide I N-H bending, amide II stretch,C=O symmetric stretch, C-N stretch and N-H out-of-plane bending respectively.

The results are thus consistent with the polymer being γ-PGA.

5.7 Summary of Bacterial Production of γ-PGA

Production of γ-PGA by two bacilli strains in GS medium and medium E was at 37°C, 150rpm for 96 hours was investigated. Table 5.3 is a summary of the results. It was observed that both strains investigated produced γ-PGA extracellularly in both media. Obtained polymers from GS medium were off-white in colour while those obtained from medium E

where brownish amorphous products. FT-IR was used to confirm the identity of the obtained polymer and GPC was used for the molecular weight analysis.

After the identification and molecular weight analysis, *B. subtilis* natto ATCC 15245 in GS medium was chosen for scale-up production in a 5l fermentation vessel for subsequent use for probiotic application. *B. subtilis* natto ATCC 15245 in GS medium was preferred as it gave the highest yield (14.11g/l) and a high molecular weight of 1,280,00. The FT-IR spectra of γ-PGA produced by *B. subtilis* natto ATCC 15245 shows similar peaks to that of commercial γ-PGA.

Table 5.3: Summary of γ-PGA (mean±SE) produced by two bacilli strains in (a) GS medium and (b) Medium E at 37°C and 150rpm for 96 hours (n=3)

GS Medium				
Strain Name	**Molecular Weight Da (M_w)**	**Molecular Number (M_n)**	**Dipersity (M_w/M_n)**	**Yield (g/l)**
B. subtilis natto ATCC 15245	1,280,000	535,000	2.4	14.11±0.09
B. lichenifomis ATCC 9945a	1,420, 000	970,000	1.5	11.03±0.07
Medium E				
Strain Name				
B. subtilis natto ATCC 15245	1,100,000	520,000	2.1	6.03±0.09
B. licheniformis ATCC 9945a	1,620,000	828,000	2.0	10.27±0.15

6.0 RESULTS - PRODUCTION OF BACTERIAL CELLULOSE

6.1 Production of BC in Different Media and Assessment of Growth of *G. xylinus*

6.1.1 Production of BC in HS medium

When *Gluconacetobacter xylinus* ATCC 23770 was grown in HS medium statically at 30°C for 14 days, an increase in cell growth from 5.36 Log CFU/ml - 6.28 Log CFU/ml was observed over 2 days (Figure 6.1), this remained constant for a further 2 days and then gradually declined over the next 10 days. BC pellicles were noticed on the air-liquid interface of the medium as a very thin film at day 4 and gradually increased in thickness to ≈10mm by day 14. From figure 6.1, it is observed that the growth curve does not follow a classic growth pattern, this is due to the movement of bacterial cells from the medium into the cellulose mat.

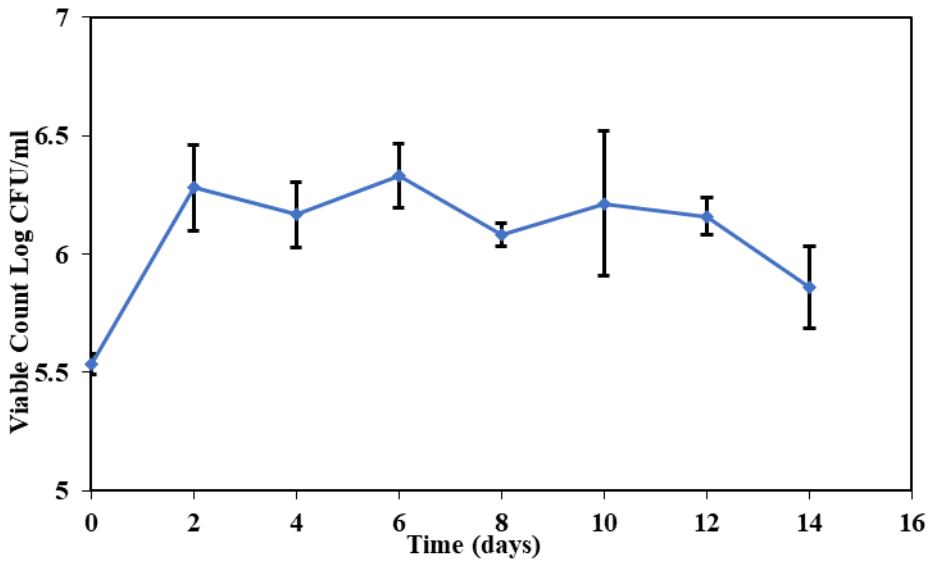


Figure 6.1: Growth curves (mean ±SE) for *G. xylinus* ATCC 23770 when grown in HS medium statically at 30°C for 14 days to evaluate the production of BC (n=3)

6.1.2 Production of BC in MHS medium

When *G. xylinus* ATCC 23770 was grown in a modified medium that contains all the components of HS medium (refer to table 4.4) with the exception of citric acid statically at 30°C for 14 days, an increase in cell growth from 5.67 Log CFU/ml to 6.17 Log CFU/ml was observed on day 2 (figure 6.2). Maximum cell number was achieved on day 6 with a viable count of 6.50 Log CFU/ml. This was followed by a constant reduction in viable count to day 14. BC pellicles were first noticed around day 4 and became thicker with time to day 14 with an average thickness of ≈14mm.

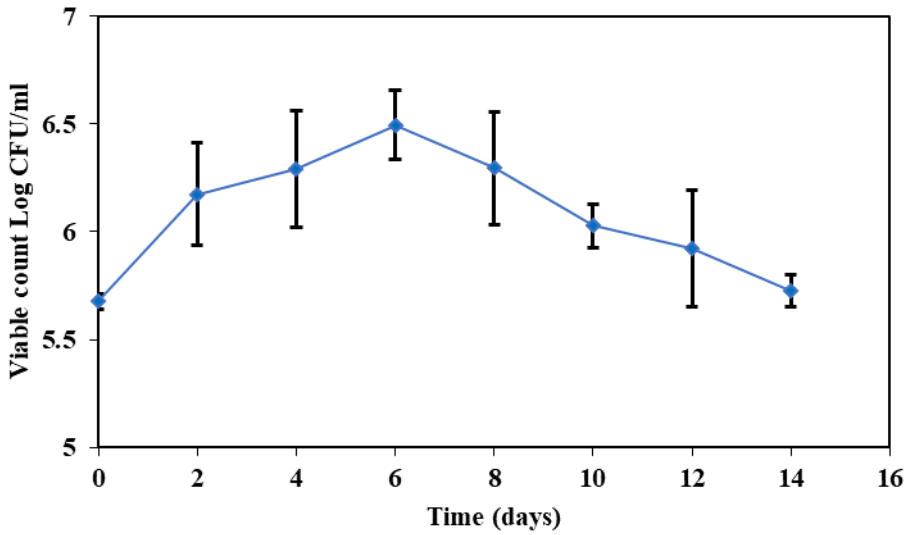


Figure 6.2: Growth curves (mean ±SE) for *G. xylinus* ATCC 23770 when grown in MHS medium statically at 30°C for 14 days to evaluate the production of BC (n=3)

6.2 Change in pH with Time

During the production of BC, it was important to investigate the change in pH of the production medium as reduction in pH has been reported due to the production of gluconic acid during fermentation. The pH of the medium was adjusted to 6.0 with glacial acetic acid and pH was monitored during the production period.

A reduction in pH was recorded in HS medium on day 2 from 6 to 5.65 (Table 6.1). Further reduction was recorded until day 10 and 12 when a slight increase in pH from 4.72 to 4.81and 4.85 was recorded on both days while the pH remained at 4.85 on day 14 when BC pellicles were harvested.

In MHS medium, a reduction in pH was recorded on day 2 from 6 to 5.88, this reduced to 4.86 on day 4 and pH remained the same on day 6. This was followed by a reduction to 4.78 on day 8; an increase to 4.81 on day 10, followed by a reduction in pH from 4.81 to 4.75 and 4.72 on days 12 and 14 respectively.

Table 6.1: Change in pH (Mean±SE) with time when *G. xylinus* ATCC 23770 was grown in HS medium and MHS medium statically at 30°C for 14 days (n=3)

Time (Days)	HS medium (pH)	MHS medium (pH)
0	6.00±0.00	6.00±0.00
2	5.65±0.03	5.88±0.01
4	4.91±0.01	4.86±0.01
6	4.86±0.03	4.86±0.02
8	4.72±0.06	4.78±0.03
10	4.81±0.01	4.81±0.01
12	4.85±0.03	4.75±0.02
14	4.85±0.00	4.72±0.00

6.3 BC Yield

BC was produced as a thick, brownish pellicle on the surface of each medium with an average thickness of ≈10mm and ≈14mm in HS and MHS media and upon purification became transparent or whitish depending on the thickness of the pellicle (figure 6.3).

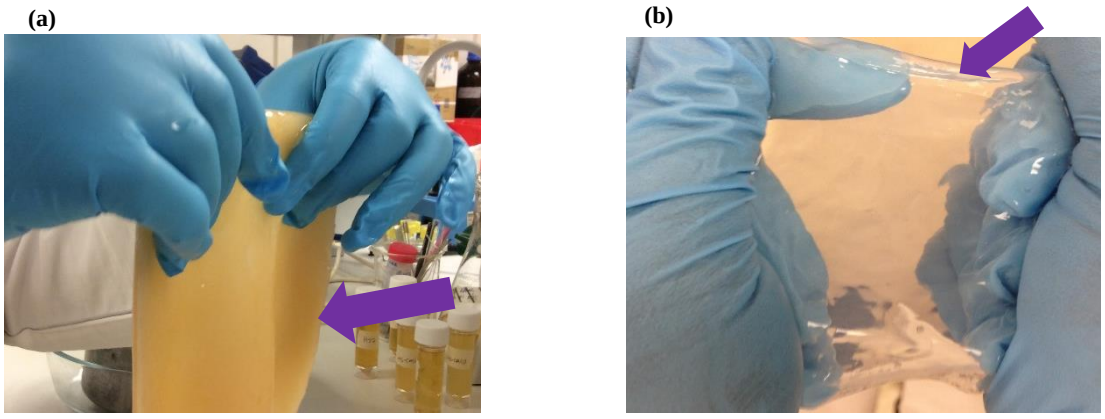


Figure 6.3: BC pellicle (a) before purification (b) post purifcation

Wet BC pellicles were patted dry with paper towels and then weighed to obtain the wet weight while dry weight was obtained after freeze-drying (Table 6.2).

An average wet weight of 31.92g/l was obtained when *G. xylinus* ATCC 23770 was grown in HS medium while an average wet weight of 78.88g/l was obtained from MHS medium. A reduction in weight was observed after freeze-drying; an average dry weight of 0.78g/l was recorded in HS medium while an average of 1.37g/l was recorded in MHS medium.

Table 6.2 Yields (mean ± SE) of BC (g/L) produced by *G. xylinus* ATCC 23770 in HS medium and MHS medium statically at 30°C for 14 days (n=3).

Medium	Average wet weight (W_w) (g/l)	Average dry weight (W_d) (g/l)
HS	31.92±1.781	0.78±0.044
MHS	78.88±4.284	1.37±0.067

The highest BC yield was obtained when *G. xylinus* ATCC 23770 was grown in MHS medium and the difference between the wet and dry weight confirms the high water holding capacity of BC.

6.4 Characterization of BC

6.4.1 FT-IR

Polymers obtained from both media were identified as cellulose using FT-IR (figure 6.4). FT-IR spectra for polymers from both HS medium and MHS medium were similar and comparable to FT-IR of standard cellulose (Sigma Aldrich, UK).

The infrared spectra of BC from both media showed strong hydroxyl absorption at 3329cm^{-1} and 3295cm^{-1}, C-H symmetrical stretch at 2878cm^{-1} and 2877cm^{-1}, a narrow OH bend of absorbed water at 1617cm^{-1} and 1629cm^{-1}, HCH and OHC in plane bending vibration at 1422cm^{-1} and 1418cm^{-1}, C-O-C asymmetrical stretch at 1121cm^{-1} and 1154cm^{-1}, and C-C, C-OH, C-H ring and side group vibrations at 1024cm^{-1} and 1028cm^{-1} for cellulose obtained from HS medium ad BC form MHS medium respectively.

6.4.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy was carried out after freeze drying. Unpurified and purified (washed with NaOH) samples were freeze-dried and examined using SEM. The SEM micrographs of BCs from both media were identical both in the unwashed and washed state, hence the SEM micrograph of BCs obtained from MHS medium is presented in this report.

The unwashed SEM micrograph (Figure 6.5a) shows BC fibres with bacterial cells embedded in the microfibrils. BC from both media showed similarity in the attachment of the cells to the microfibrils of the cellulose. Figure 6.5b shows the SEM micrograph of purified BC from MHS medium.

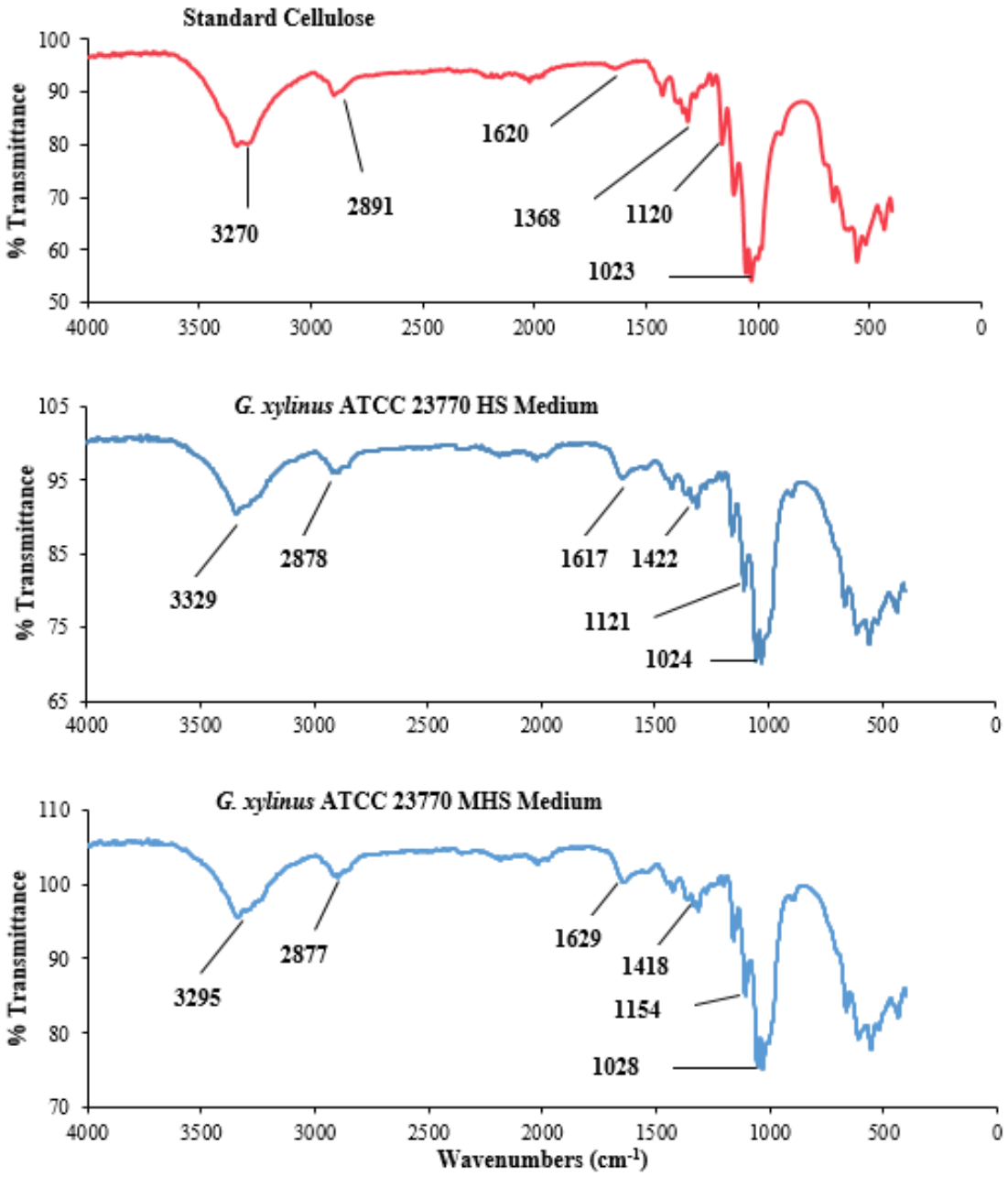


Figure 6.4: FT-IR spectra of BCs produced by *G. xylinus* ATCC 23770 in HS medium and MHS medium statically at 30°C for 14 days compared with the spectra of standard cellulose. Each spectrum is an average of 3 spectra (n=3)

After purification, bacterial cells have been removed from the cellulose fibres as seen in figure 6.5b, it also reveals tightly packed microfibrils in BC from both media. The process of washing BC pellicles in hot NaOH results in the removal of bacteria and other materials to reveal a network of microscopic fibres.

(a)

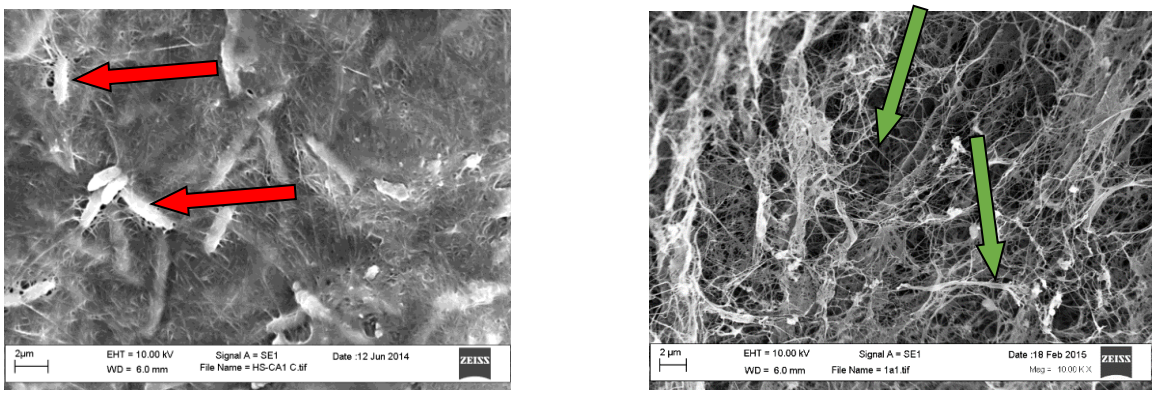


Figure 6.5: SEM images of (a) unwashed BC with bacterial cells attached to the cellulose fibres and (b) purified BC revealing the tightly packed microfibrils from MHS medium. BC was produced by *G. xylinus* ATCC 23770 statically at 30°C for 14 days (red arrows indicate bacterial cells attached to cellulose fibres, green arrows indicate cellulose fibres)

6.4.3 X-Ray Diffraction (XRD)

X- ray diffraction results obtained from the BC samples from HS medium and MHS medium show clearly resolved peaks (Figures 6.6a and b) which are similar in both BC samples. X-ray diffractogram thus indicates that the cellulose produced from both media were crystalline in nature, typical of type 1 cellulose.

6.5 Milling of BC

For the probiotic application, dried BC sheets were milled into powdered form (PBC). Since a high yield was obtained when *G. xylinus* ATCC 23770 was grown in MHS medium, cellulose from this medium was chosen for the probiotic application. Freeze-dried samples were initially ground using liquid nitrogen and a laboratory mortar and pestle, however, this method was not suitable as samples came out flaky and not powdery.

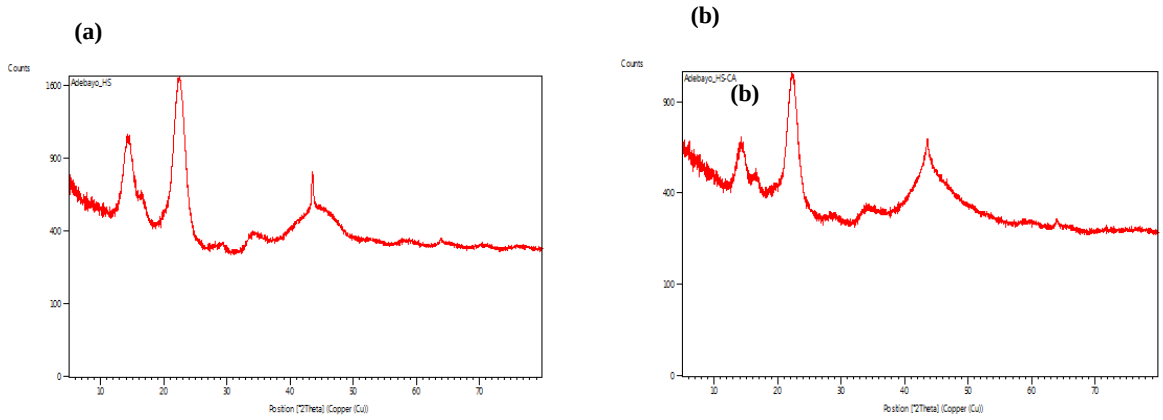


Figure 6.6: XRD result of BC produced when *G. xylinus* ATCC 23770 was grown in (a) HS and (b) MHS media statically at 30°C for 14 days

Samples were then milled using a Pulverisette 5 (Fritsch, Germany) machine at the Telford Innovation campus of the University of Wolverhampton, but this method was also not suitable as samples turned black due to the heat and due to the grinding of the balls in the bowl.

Consequently, samples were then sent to C. Gerhardt Limited, UK for milling using the variable speed rotor mill Pulverisette 14 (Fritsch, Germany) fitted with an 0.08mm sieve ring.

To investigate if the milling had any detrimental effect on the unique characteristic features of BC, PBC was analysed using FT-IR, SEM and XRD. Figure 6.7 shows the FT-IR of PBC compared with BC sheet obtained from *G. xylinus* ATCC 23770 in MHS medium.

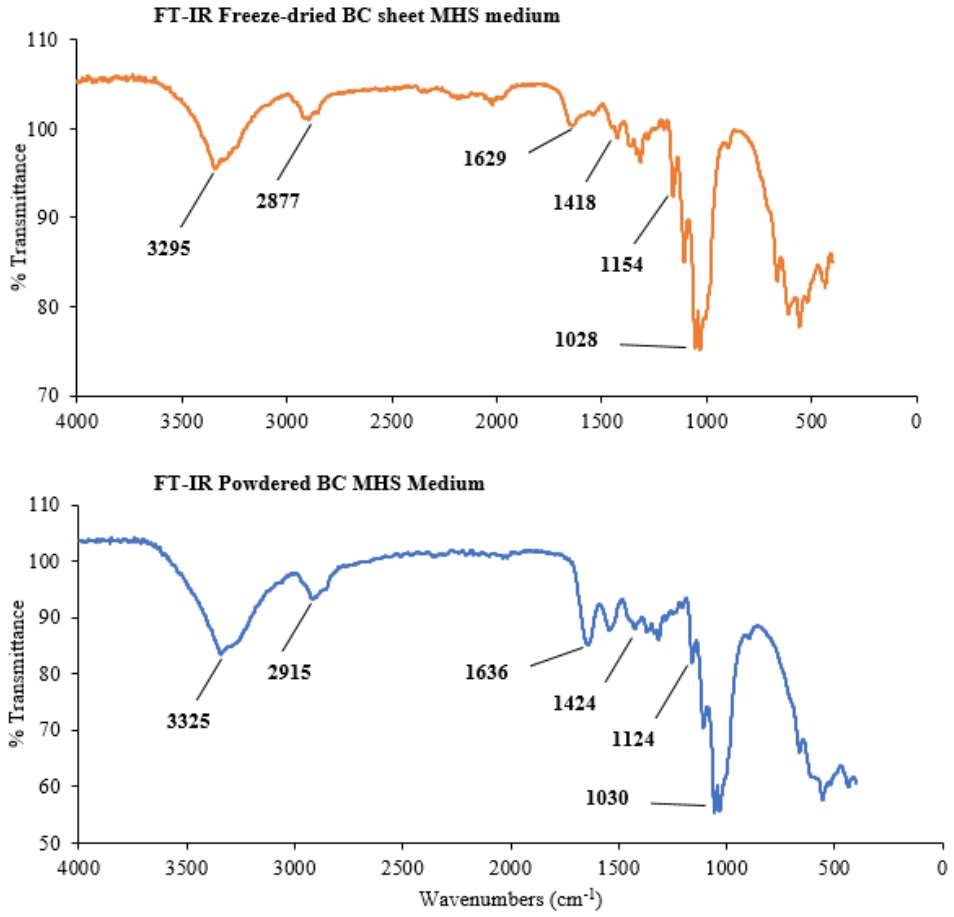


Figure: 6.7: FT-IR spectra of freeze-dried BC sheets from MHS medium and Powdered BC from *G. xylinus* ATCC 23770 grown statically in MHS medium at 30° for 14 days

FT-IR spectra obtained from PBC and BC sheet are comparable but with PBC showing a more distinct OH bend of absorbed water at 1636cm^{-1}.

Figure 6.8 shows the SEM image of PBC (a) compared with BC sheet (b)

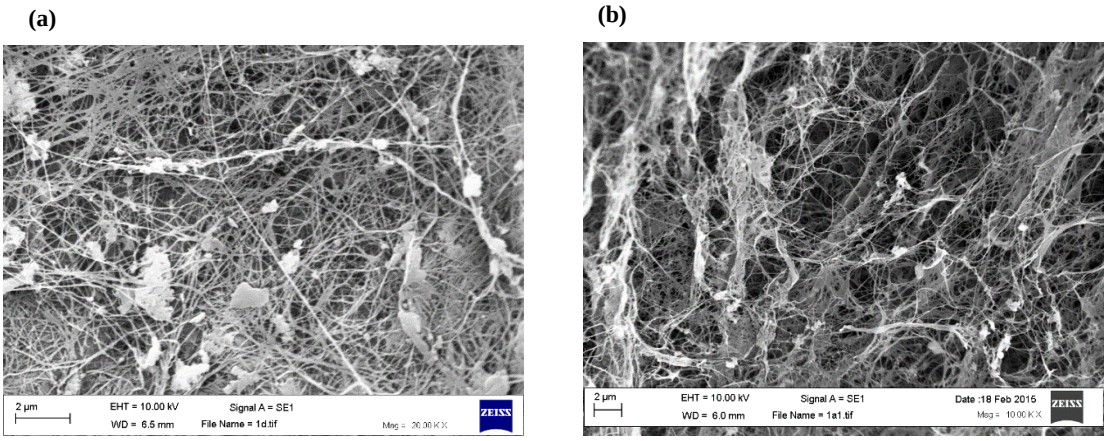


Figure 6.8: SEM micrographs of (a) PBC and (b) BC sheet from MHS medium from *G. xylinus* ATCC 23770 grown statically in MHS medium at 30°C for 14 days

SEM micrograph of PBC reveal tightly packed microfibrils similar to that of BC sheet from MHS medium. Result from the XRD analysis reveals the crystallinity of PBC as seen in figure 6.12.

Results from the different analyses of PBC revealed the milling process had no detrimental effect on the unique characteristics of bacterial cellulose such as the crystallinity and the microfibrillar network as the results obtained were comparable to BC sheets.

6.6 Summary of BC Production

Bacterial cellulose was produced from *G. xylinus* ATCC 23770 using HS medium and a modified medium (MHS). *G. xylinus* ATCC 23770 was grown statically at 30°C for 14 days and BC pellicles were noticed as thick pellicles with an average thickness of about 14-18mm floating on the medium. BC pellicles were harvested; purified, freeze-dried and analysed.

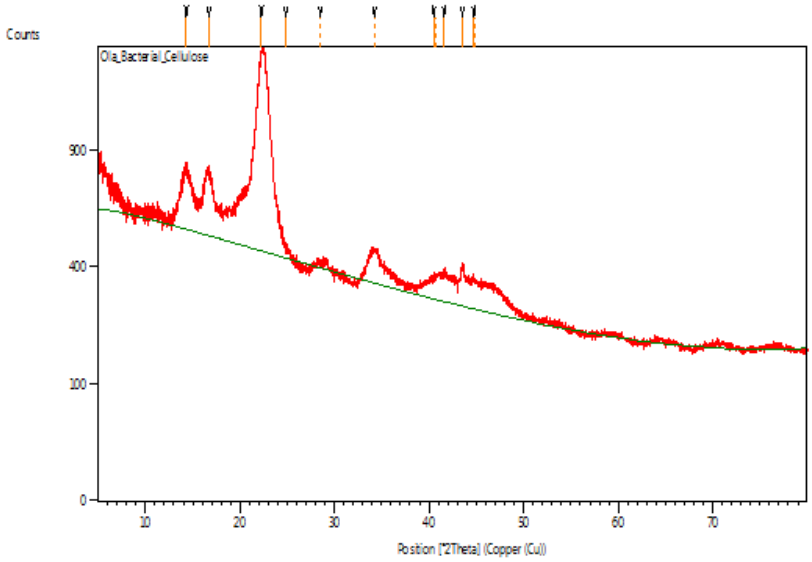


Figure 6.9: XRD result of PBC from MHS medium from *G. xylinus* ATCC 23770 grown statically in MHS medium at 30°C for 14 days

Higher BC yield (wet weight 78.88g/l, dry weight 1.37g/l) was obtained when *G. xylinus* ATCC 23770 was grown in the modified medium. Obtained BCs were analysed using FT-IR, SEM and XRD. FT-IR results reveal similarities in the functional groups of the produced BCs and standard cellulose, SEM micrographs show similarities in the arrangement of the microfibrils of BCs from both media. XRD also reveals the crystallinity of produced BCs.

Powdered BC produced was also analysed to investigate if the milling process had any detrimental effect on the unique characteristics of BC, the process of milling had no detrimental effect on the features of BC as the results obtained from the analyses of PBC were comparable to results obtained from BC.

7.0 RESULTS – PROBIOTIC STUDIES

7.1 Comparing the Effect of Known Cryoprotectants with BC, PBC and γ-PGA on Probiotic Strains on Bifidobacteria during Freeze-Drying

7.1.1 Immobilization of Bifidobacteria *breve* NCIMB 8807 and *B. longum* NCIMB 8809 in Wet BC Sheets

Wet BC sheets were cut into small dices of about 5mm. Cut cubes of BC were then sterilised by autoclaving at 0.35 bar at 110°C for 30 minutes. An average viable count of 10^9 CFU/ml was recorded in both strains and approximately 20g sterile wet BC sheets were then added to probiotic culture medium (*B. longum* NCIMB 8809 and *B. breve* NCIMB 8807); incubated anaerobically for 24 hours, freeze-dried and SEM was used to investigate the attachment of probiotic cells to the BC fibre network.

During this study, estimating the viable count after freeze drying was challenging as cells could not be dislodged from cellulose fibres. However, SEM analyses reveal the attachment of probiotic bacteria to the fibre network of BC. BC network provide a means of support for probiotic strains during free-drying as seen in figure 7.2. The control sample (cut cubes of BC in BSM broth) which do not contain any probiotic strain shows no cells attached (Figure 7.2c).

7.1.2 Cryoprotective Effects of γ-PGA and PBC Compared with Sucrose and Skimmed Milk Powder

The cryoprotective effects of 5% γ-PGA and 5% PBC were compared with the cryoprotective effect of known cryoprotectants- 5% sucrose and 5% skimmed milk powder (SMP) on six strains of bifidobacteria- *B. breve* NCIMB 8807, *B. longum* NCIMB 8809, *B. adolescentis* NCIMB 702229, *B. animalis* NCIMB 702716, *B. infantis* NCIMB 702255 and *B. bifidum* NCIMB 702715. The results are presented in figure 7.1 (a-f).

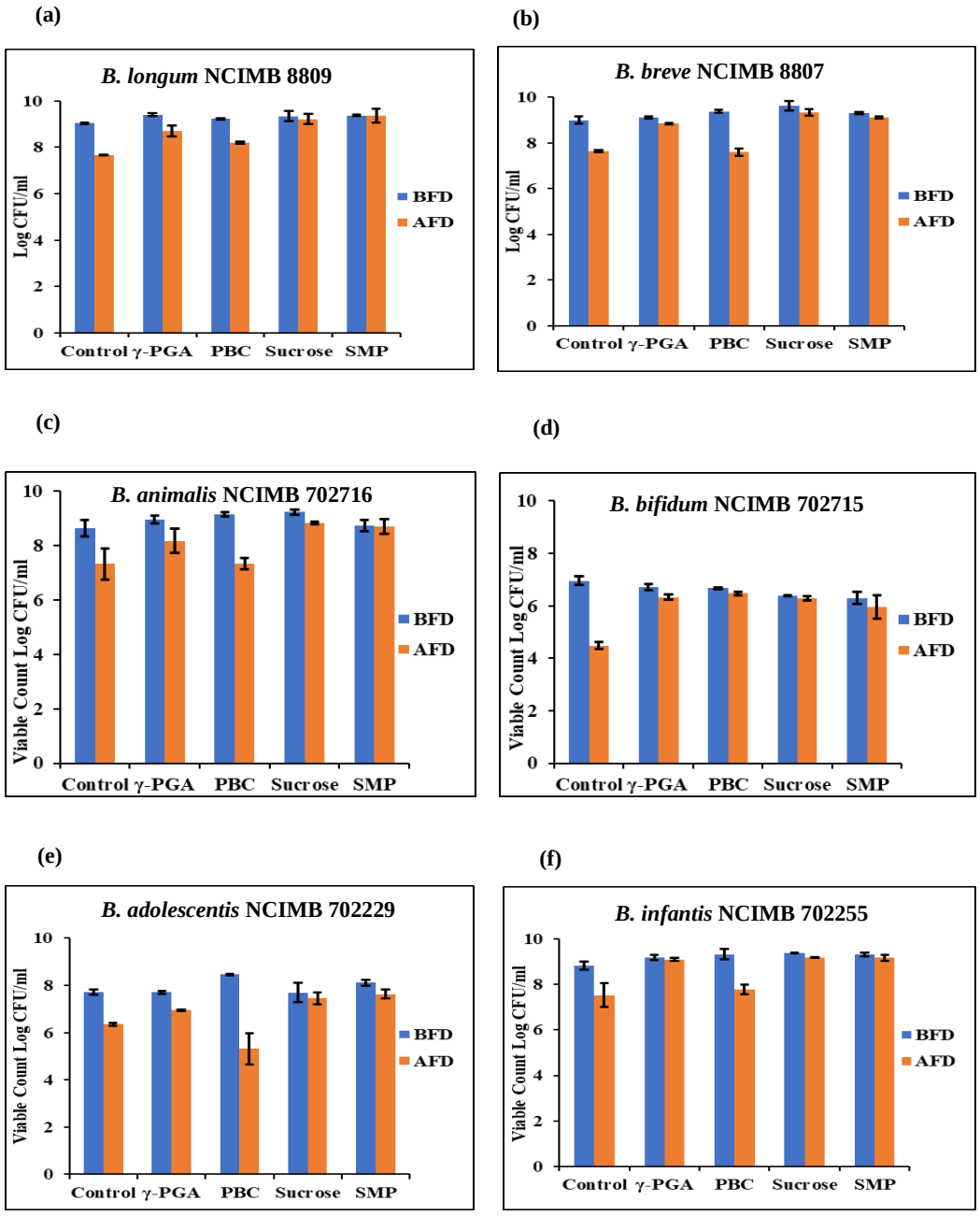


Figure 7.1: Cryoprotective effect of 5% γ-PGA, 5% PBC, 5% SMP and 5% sucrose on viability of (a) *B. longum* NCIMB 8809, (b) *B. breve* NCIMB 8807, (c) *B. animalis* NCIMB 702716, (d) *B. bifidum* NCIMB 702715, (e) *B. adolescentis* NCIMB 702229 and (f) *B. infantis* NCIMB 702255 during freeze drying. Cells were freeze-dried at -40°C and 5 Mbar pressure. Viability was measured before freeze drying (BFD) and after freeze drying (AFD), plated on BSM agar plates and incubated anaerobically for 48 hours at 37°C. Control samples contained no added cryoprotectant (±SE, n-3). SMP is skimmed milk powder and PBC is powdered bacterial cellulose

When cells were not protected with any cryoprotectant, a reduction of about 1.36 Log CFU/ml was observed in all investigated probiotic strains (figure 7.2) except for *B. bifidum* NCIMB 702715 where a loss of 2.48 Log CFU/ml was recorded after freeze drying. The mean values of viable cells in the unprotected cells before and after freeze drying were significantly different and not comparable ($p<0.05$).

When cells were protected with 5% γ-PGA, a reduction of about 0.76 Log CFU/ml was recorded in all six strains after freeze drying. With 5% PBC, the maximum reduction in cell viability (3 Log) was recorded in *B. adolescentis* NCIMB 702229 while the lowest loss (0.2 Log) in cell viability after freeze drying was recorded in *B. bifidum* NCIMB 702715.

When cells were protected with sucrose, the lowest loss in cell viability (0.10 Log CFU/ml) after freeze drying was recorded in *B. bifidum* NCIMB 702715, the highest loss in cell viability (0.38 Log CFU/ml) was recorded in *B. infantis* NCIMB 702255. When cells were protected with SMP, the lowest viable loss (0.01 Log CFU/ml) was recorded in *B. longum* NCIMB 8809 while the highest loss in viability (0.49 Log CFU/ml) after freeze drying was recorded in *B. adolescentis* NCIMB 702229.

The cryoprotective effect of γ-PGA was comparable to the protective effect of the known cryoprotectants (sucrose and SMP) and there was no significant difference between the mean viable cells after freeze drying in cells protected with γ-PGA, sucrose and SMP ($P>0.05$). There was no significant difference ($p>0.05$) between the cryoprotective effect of γ-PGA and PBC in *B. bifidum* NCIMB 702715 and *B. animalis* NCIMB 702716, however, other strains behaved differently and there was a significant difference ($p<0.05$) between the cryoprotective effect of γ-PGA and PBC.

The cryoprotective effect of PBC compared with the cryoprotective effect of sucrose and SMP was comparable and there was no significant difference ($p>0.05$) in *B. animalis* NCIMB

7027156 and *B. bifidum* NCIMB 702715 while in the other strains, there was a significant difference ($p<0.05$) between the cryoprotective effect of PBC and sucrose and SMP. Generally, PBC was less effective as a cryoprotectant compared with γ-PGA.

Generally, the optimal cryoprotectant for preventing loss of viability depends upon the bacterial species being tested. Table 7.1 summarises these results.

Table 7.1: Optimal cryoprotectant for preventing loss of viability in selected bifidobacteria strains

Bifidobacteria Strain	Optimal Cryoprotectant
B. longum NCIMB 8809	Skimmed milk powder
B. breve NCIMB 8807	Skimmed milk powder
B. animalis NCIMB 702716	Skimmed milk powder
B. adolescentis NCIMB 702229	Sucrose
B. bifidum NCIMB 702715	Sucrose
B. infantis NCIMB 702255	γ-PGA

7.1.3 SEM Analysis

Scanning electron microscopy was used to analyse and understand how cells are protected by wet BC sheets, PBC, γ-PGA and SMP. It was difficult analysing sucrose due to its high water absorbing capacity after freeze drying. Freeze dried powders containing unprotected *B. breve* NCIMB 8807 and *B. breve* NCIMB 8807 protected with γ-PGA, PBC and SMP, and freeze-dried BC sheets containing *B. breve* NCIMB 8807 were analysed. The SEM analysis (figure 7.2a) of unprotected *B. breve* NCIMB 8807 cells show free cells clustered around each other; figure 7.2b shows BC sheet with no cells attached to it; the SEM analysis of cells immobilised with BC sheet shows the attachment of probiotic cells to the fibre network of BC (Figure 7.2c) while the SEM analysis of cells protected with PBC (figure 7.2d) reveals cells embedded within the fibre network structure of cellulose.

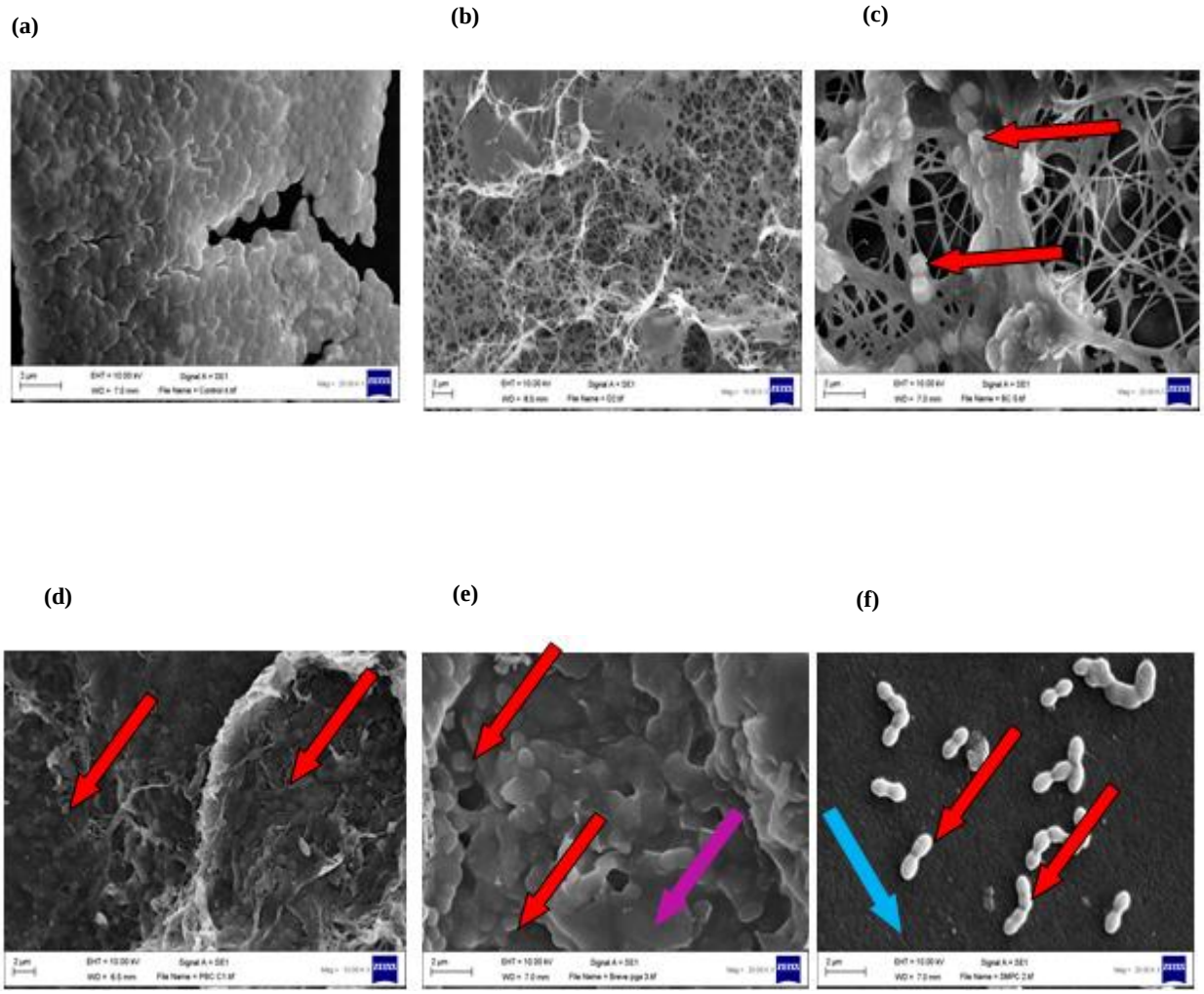


Figure 7.2: SEM image of (a) freeze dried *B. breve* NCIMB 8807 cells with no protection, (b) freeze dried BC sheet with no cells attached, (c) freeze dried *B. breve* NCIMB 8807 cells immobilised in BC sheets (d) freeze dried *B. breve* NCIMB 8807 cells protected with PBC, (e) freeze dried *B. breve* NCIMB 8807 cells protected with γ-PGA and (f) freeze dried *B. breve* NCIMB 8807 cells protected with skimmed milk powder(SMP). Red arrows indicate bacterial cells while purple and blue arrows indicate γ-PGA and SMP matrix respectively. SEM analysis was performed using Zeiss EVO50, UK

The SEM analyses of γ-PGA (figure 7.2e) and SMP (figure 7.2f) protected cells reveal cells to be encapsulated within the matrices of γ-PGA and SMP.

7.2 Antimicrobial Properties of Bifidobacteria Strains on Selected Pathogenic Bacteria

Probiotic strains have been reported to have antimicrobial activities, and reports have shown that the antimicrobial activities vary between different strains. This led to investigating if the

selected strains have antimicrobial activities against 4 pathogenic strains (*L. innocua* NCTC 11288, *S. aureus* NCIMB 6571, *S. typhimurium* WLV 73 Cardiff Collection and *E. coli* W1485-K12 W-T).

During this investigation, probiotic strains were grown anaerobically in TPY medium (pH 6.5) at 37°C, 150rpm and for 24 hours. Cells were then centrifuged to obtain cell free culture supernatant (CFCS). The pH of the CFCS was recorded (table 7.2).

Table 7.2: pH of CFCS obtained from the probiotic strains prior to adjusting the pH to 7. Probiotic strains were grown anaerobically at 37°C, 150rpm for 24 hours. Initial pH of medium before incubating 6.5

Probiotic Strain	pH of CFCS
B. longum NCIMB 8809	4.47±0.41
B. breve NCIMB 8807	4.33±0.28
B. animalis NCIMB 702716	4.58±0.10
B. adolescentis NCIMB 702229	5.92±0.65
B. bifidum NCIMB 702715	5.97±0.34
B. infantis NCIMB 702255	5.28±0.63

pH of 3 replicates ± SE

All the selected probiotic strains showed a reduction in pH, pH ranged between 5.97-4.33. The lowest pH (4.33±0.28) was recorded for *B. breve* NCIMB 8807 while the highest pH (5.97±0.34) was recorded for *B. bifidum* NCIMB 702715. The reduction in pH is likely due to the production of acetic acid and lactic acids from glucose fermentation which is the main carbohydrate source in TPY medium.

CFCS were then grouped into two; the first group contained the original CFCS while the pH of the second group was adjusted to 7 and all samples were filter-sterilised using a 0.22µm-pore size micro filter.

The inhibitory effect of the two groups of CFCS on the selected pathogenic strains was then investigated (refer to section 4.3.6.2) . The zones of inhibition were recorded in the non-concentrated samples and in concentrated samples and results are presented in table 7.3 and 7.4.

When the inhibitory effect of non-adjusted pH CFCS of probiotic strains against selected human pathogens was investigated, *B. longum* NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 showed significant inhibition against all selected pathogens both in the concentrated and non-concentrated states. *B. bifidum* NCIMB 702715 demonstrated inhibitory effect on all pathogens excluding *S. aureus*, however, the concentrated CFCS from *B. bifidum* NCIMB 702715 showed inhibitory effect on the growth of *S. aureus* NCIMB 6571.

In the concentrated state, CFCS from *B. infantis* NCIMB 702255 demonstrated inhibitory effect against all pathogens except *S. aureus* NCIMB 6571, while the non-concentrated CFCS from *B. infantis* NCIMB 702255 did not show any inhibitory effect against all pathogens.

The concentrated CFCS from *B. adolescentis* NCIMB 702229 showed an inhibitory effect against the growth of *S. aureus* NCIMB 6571 while there was no inhibitory effect on other pathogens both in the concentrated and non-concentrated forms.

At pH 7, non-concentrated CFCS from the probiotic strains showed no inhibition against all selected pathogens (Table 7.4) indicating that low pH might be responsible for inhibitory effect. However, when CFCS was concentrated; *B. longum* NCIMB 8809 demonstrated inhibition to the growth of all pathogens and *B. breve* NCIMB 8807 inhibited the growth of all pathogens except *S. aureus* NCIMB 6571. *B. animalis* NCIMB 702716 and *B. bifidum* NCIMB 702715 inhibited the growth of *E. coli* W1485-K12 W-T while *B. adolescentis* NCIMB 702229 and *B. infantis* NCIMB 702255 inhibited the growth of *S. aureus* NCIMB

6571. These results indicate that some other antimicrobial agent(s) have been released into the medium during growth of probiotic bacteria.

There was no significant change in the zones of inhibition in all selected strains at 48 hours compared with the zones of inhibition at 24 hours.

Table 7.3: Inhibitory effect of non-adjusted pH CFCS of probiotic strains against selected pathogens using the well diffusion assay.

	***L. innocua* NCTC 11288**		***S. aureus* NCIMB 6571**		***E. coli* W1485-K12 W-T**		***S. typhimurium* WLV 73 Cardiff Collection**	
Probiotic Strain	**NC**	**C**	**NC**	**C**	**NC**	**C**	**NC**	**C**
***B. longum* NCIMB 8809**	7.3±0.06	12.5±0.05	11.00±0.15	10.3±0.05	13.8±0.14	11.5±0	8.80±0.13	10.60±0.09
***B. breve* NCIMB 8807**	11.00±0.02	12.80±0.07	13.4±0.44	9.60±0.04	11.80±0.13	9.00±0.06	9.50±0.18	9.80±0.07
***B. animalis* NCIMB 702716**	10.3±0.03	11.5±0.03	10.8±0.03	10.2±0.1	13.00±0.13	15.8±0.22	10.00±0.10	10.3±0.06
B. adolescentis	X	x	11.70±0.04	X	x	x	x	x
***B. bifidum* NCIMB 702715**	10.8±0.13	8.50±0	x	11.3±0.03	9.5±0.15	9.3±0.08	10.00±0.05	8.3±0.03
***B. infantis* NCIMB 702255**	X	8.50±0	x	X	x	7.0±0	x	7.0±0

Plates were incubated overnight at 37°C and zones of inhibitions measured in mm. The diameter of the zones of inhibition were measured, expressed in mm and were an average of three replicates ±SE (n=3). (NC: Non-concentrated, C: Concentrated, x: no inhibition)

Table 7.4: Inhibitory effect of pH adjusted (pH was adjusted to 7 with 3M NaOH) CFCS of probiotic strains against selected pathogens using the well diffusion assay.

	***L. innocua* NCTC 11288**		***S. aureus* NCIMB 6571**		***E. coli* W1485-K12 W-T**		***S. typhimurium* WLV 73 Cardiff Collection**	
Probiotic Strain	**NC**	**C**	**NC**	**C**	**NC**	**C**	**NC**	**C**
***B. longum* NCIMB 8809**	X	10.80±0.03	x	10.3±0.04	X	11.5±0	X	10.6±0.09
***B. breve* NCIMB 8807**	X	11±0	x	X	X	8.00±0.03	X	10.00±0
***B. animalis* NCIMB 702716**	X	X	x	X	X	7.00±0	X	x
***B. adolescentis* NCIMB 702229**	X	X	x	11.3±0.20	X	x	X	x
***B. bifidum* NCIMB 702715**	X	X	x	X	X	8.5±0	X	x
***B. infantis* NCIMB 702255**	X	X	x	1.05±0	X	x	X	x

Plates were incubated overnight at 37°C and zones of inhibitions measured in mm. The diameter of the zones of inhibition were measured, expressed in mm and were an average of three replicates ±SE (n=3). (NC: Non-concentrated, C: Concentrated, x: no inhibition)

7.2.1 Organic Acid Production

Probiotic strains were further investigated for organic acid produced during cultivation as a consequence of the antimicrobial test results (refer to section 7.2).

The cell growth and pH were measured with time with the strains showing varying growth patterns and a reduction in pH was observed in most of the strains investigated during the 48-hour period (Figure 7.3).

B. longum NCIMB 8809 achieved maximum cell numbers at 24 hours with an average cell count of 8.95 Log CFU/ml, at 8 hours a reduction in pH from 6.50 to 4.53 and a further reduction to pH 4.26 at 24 hours. At 48 hours, reduction in cell count (8.5 Log CFU/ml) was recorded, this was accompanied with a further reduction in pH to pH 4.17 (figure 7.3a).

When *B. breve* NCIMB 8807 was grown in TPY, an initial cell count of 5.94 Log CFU/ml was recorded, this was followed by a 3 Log increase at 8 hours, accompanied by a reduction in pH from pH 6.50 - pH 4.56. Maximum cell count of 8.75 Log CFU/ml was achieved after 24 hours, with a further reduction in pH to pH 4.20 followed by a slight reduction (8.42 Log CFU/ml) in cell count after 48 hours and a further reduction in pH to pH 4.13 (figure 7.3b).

In *B. animalis* NCIMB 702716 (figure 7.3c), a slight increase in cell population was observed after 8 hours (6.61 Log CFU/ml - 7.15 Log CFU/ml), this was accompanied by a reduction in pH to pH 4.6. Maximum cell population was achieved after 24 hours (8.9 Log CFU/ml) and a further reduction in pH (4.2) was recorded. After 48 hours, slight reductions in cell population (8.52 Log CFU/ml) and pH (4.05) were recorded.

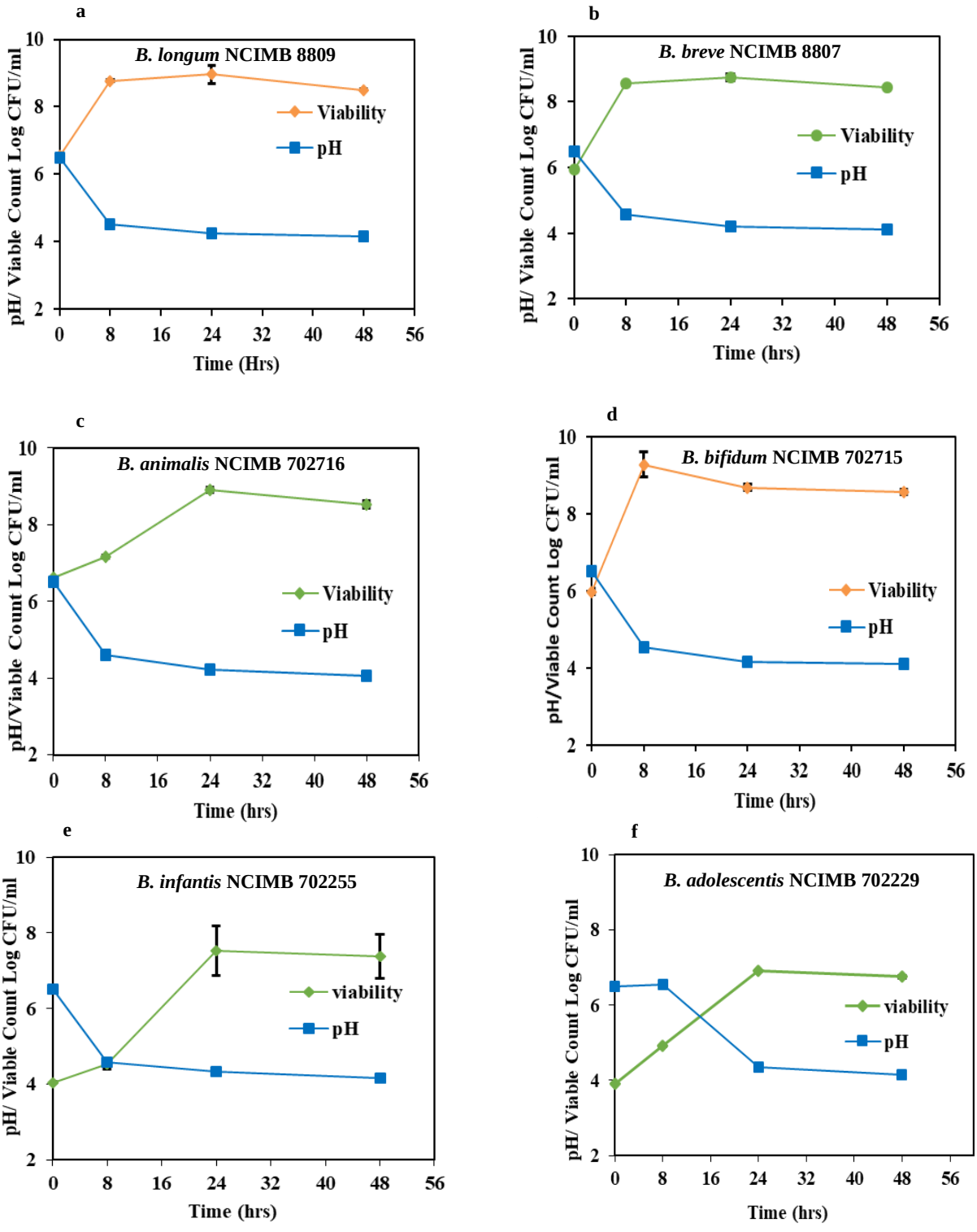


Figure 7.3: Growth curve and pH measurement (mean ±SE) for all investigated bifidobacteria strains: (a) *B. longum* NCIMB 8809, (b) *B. breve* NCIMB 8807, (c) *B. animalis* NCIMB 702716, (d) *B. bifidum* NCIMB 702715, (e) *B. infantis* NCIMB 702255 and (f) *B. adolescentis* NCIMB 702229 when grown in TPY medium anaerobically at 37°C, 150 rpm for 48 hours (n=3).

When *B. bifidum* NCIMB 702715 (figure 7.3d) was grown in TPY medium for 48 hours, maximum cell population (9.27 Log CFU/ml) was achieved after 8 hours as well as a reduction in pH 4.53. This was followed by reductions in cell population (8.68 Log CFU/ml) and pH (4.17) after 24 hours. Slight reductions in cell population (8.56 Log CFU/ml) and pH (pH 4.10) were also recorded after 48 hours.

B. infantis NCIMB 702255 (figure 7.3e) showed slower growth compared with other strains at 8 hours, a slight increase was observed from 4.04 Log CFU/ml - 4.54 Log CFU/ml, however, reduction in pH was also observed at this time (pH 4.58). Maximum cell population (Log CFU/ml) was achieved in *B. infantis* NCIMB 702255 after 24 hours, this was accompanied by a reduction in pH to pH 4.34. A slight reduction in cell population (7.38 Log CFU/ml) was recorded after 48 hours and a slight reduction in pH (pH 4.17).

When *B. adolescentis* NCIMB 702229 was grown in TPY medium, increase in cell population was also slow compared with the other strains investigated (figure 7.3f) with an increase from 3.90 Log CFU/ml - 4.91 Log CFU/ml and this correlates with the change in pH as the pH after 8 hours (pH 6.55) remained almost the same as the initial pH. However, similar to other strains under investigation, maximum cell population (6.91Log CFU/ml) was achieved after 24 hours with a reduction in pH to pH 4.36. Slight reductions in cell population (6.76 Log CFU/ml) and pH (pH 4.15) were recorded after 48 hours.

7.2.2 Organic Acid Assay

Lactic and acetic acids are known to be produced during the fermentation of probiotic strains in culture medium. CFCS obtained from the selected probiotic strains were investigated for the concentration of organic acids produced during the fermentation period and the Megazyme assay kit was used for this investigation (refer to section 4.3.6.3). During the investigation; it was discovered that acetic acid was produced in low concentrations ranging

from 0.01g/l at zero hour to about 0.14g/l at 48 hours with *B. breve* NCIMB 8807 having the highest concentration of 0.14g/l at 48 hours. However, lactic acid was produced by all the selected probiotic strains in concentrations ranging from 0.01g/l at zero hours to ≈ 1.76g/l at 48 hours in the selected strains.

The total lactic acid concentrations at zero hour in all the selected strains was ≈ 0.01g/l (figure 7.4). At 8 hours, all the selected strains (excluding *B. adolescentis* NCIMB 702229) showed significant changes in the concentration of total lactic acid (figure 7.4). *B. infantis* NCIMB 702255 had the highest lactic acid concentration of 1.015g/l in the CFCS at 8 hours while *B. adolescentis* NCIMB 702229 had the least concentration of 0.03g/l.

At 24 hours, *B. longum* NCIMB 8809 had the highest amount of total lactic acid concentration of 1.56g/l, followed by *B. bifidum* NCIMB 702715 and *B. animalis* NCIMB 702716 with total lactic acid concentrations of 1.52g/l. *B. breve* NCIMB 8807 had a total lactic acid concentration of 1.45g/l; *B. infantis* NCIMB 702255 had a total lactic acid concentration of 1.23g/l while the lowest total lactic acid concentration (0.26g/l) was recorded in *B. adolescentis* NCIMB 702229.

At 48 hours, all the selected strains showed a further increase in total lactic acid concentration. *B. longum* NCIMB 8809 had the highest total lactic acid concentration (1.76g/l) while *B. adolescentis* NCIMB 702229 had the least total lactic acid concentration (0.56g/l).

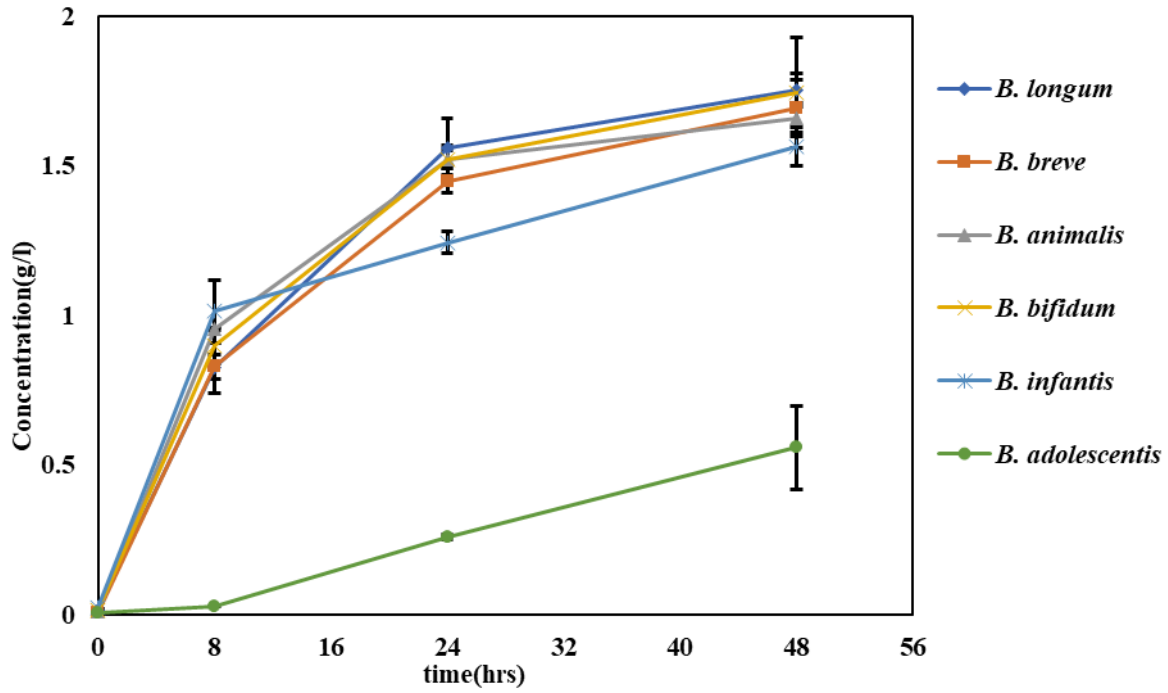


Figure 7.4: Total lactic acid concentration (±SE) present in cell free culture supernatants of *B. longum* NCIMB 8809, *B. breve* NCIMB 8807, *B. animalis* NCIMB 702716, *B. bifidum* NCIMB 702715, B. *infantis* NCIMB 702255 and *B. adolescentis* NCIMB 702229 over 48 hours, cells were grown anaerobically in TPY medium at 37°C and 150rpm (n=3)

7.2.3 Effect of Lactic Acid upon the Growth of Selected Pathogens

Lactic acid is the major organic acid produced by all selected probiotic strains; hence, the effect of commercial lactic acid on the growth of pathogens was investigated. The highest concentration of total lactic acid in the selected probiotics strains at 24 hours is 1.56g/l which was obtained from the CFCS of *B. longum* NCIMB 8809. This concentration (1.56g/l) was used for this investigation and different concentrations of lactic acid (0.5g/l, 1g/l and 2g/l) were used for comparison.

The well diffusion assay method was used for this investigation and there was no inhibition of the pathogens with 0.5g/l, 1g/l and 1.56g/l lactic acid; however, inhibition was observed with 2g/l lactic acid in *S. typhimurium* WLV 73 Cardiff Collection and *S. aureus* NCIMB

6571, while no inhibition was recorded in *L. innocua* NCTC 11288 and *E. coli* W1485-K12 W-T.

This result shows there are other factors responsible for the inhibitory properties exhibited by the selected probiotic strains since no inhibition was observed when the same concentration of lactic acid obtained from the CFCS of the investigated probiotic strains was used.

7.3 Biopolymer Protection of Probiotic Bacteria in Simulated Gastro Intestinal Conditions

For protection of probiotic bacteria in simulated intestinal conditions, the following strains were used: *B. breve* NCIMB 8807; *B. longum* NCIMB 8809 and *B. animalis* NCIMB 702716. The protective effect of 5% and 2.5% γ-PGA; 2.5% and 5% PBC and a combination of γ-PGA and PBC [2.5% (w/v) PBC and 2.5% (w/v) γ-PGA] was investigated when these probiotic cells were exposed to simulated intestinal conditions. Simulated intestinal conditions examined include exposure to simulated gastric juice, simulated intestinal juice and exposure to bile salts (Refer to section 4.3.7 for the composition of these intestinal juices).

7.3.1 Protection in Simulated Gastric Juice (SGJ)

B. longum NCIMB 8809

The effects of 5% γ-PGA, 5% PBC and γ-PGA + PBC (2.5% PBC and 2.5% γ-PGA) were tested on the viability of *B. longum* NCIMB 8809 in SGJ for four hours (figure 7.5). In the control samples (unprotected cells), a decline in cell viability was recorded at time 1hr (4.03 Log CFU/ml) which was followed by total loss in viability by 2 hours.

When 5% γ-PGA was used to protect *B. longum* NCIMB 8809 in SGJ, a total loss of 0.27 Log CFU/ml was recorded after four hours ($p<0.05$). When 5% PBC was used to protect *B.*

longum NCIMB 8809 in SGJ; a total loss of 2.49 Log CFU/ml was recorded after four hours, statistical analysis reveals a significant difference between the number of cells at 0 hr and 4hrs ($p<0.05$).

When the combination of γ-PGA and PBC was used to protect *B. longum* NCIMB 8809 in SGJ, a total loss of 0.47 Log CFU/ml was recorded after 4 hours. The number of cells at 0hr and 4hrs are comparable and were not significantly different ($p>0.05$).

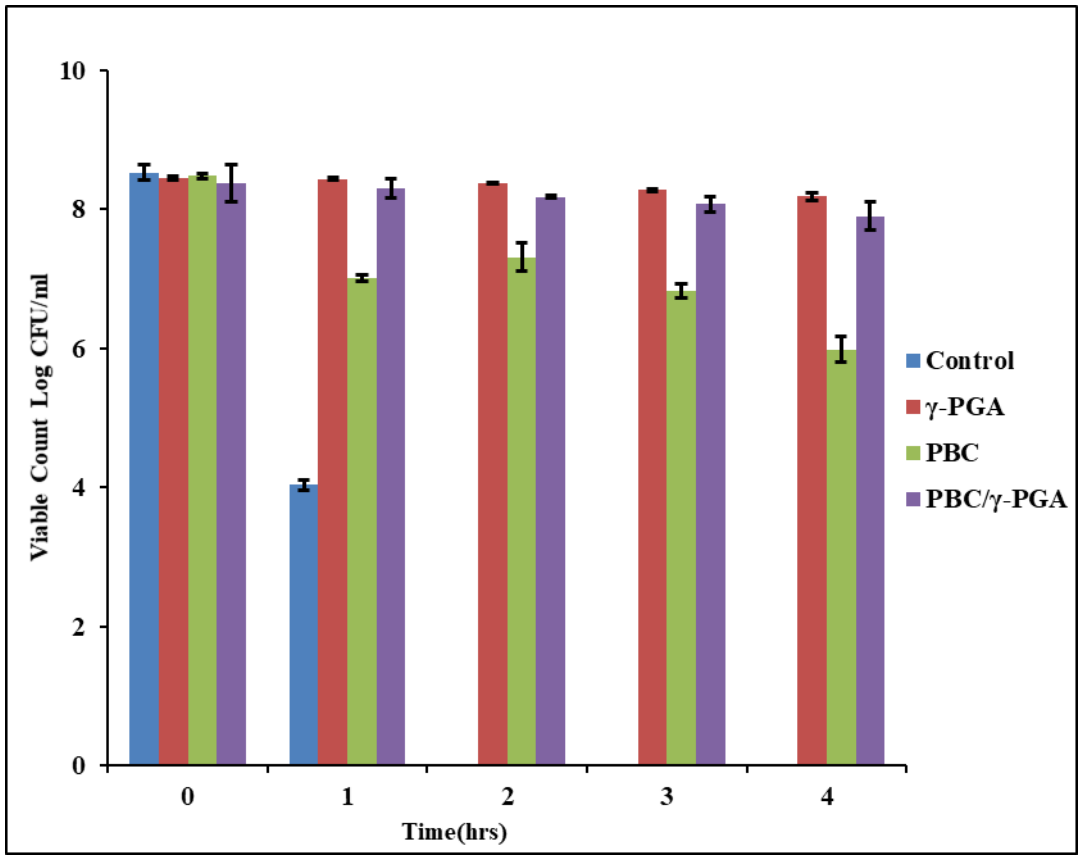


Figure 7.5: Protective effect of 5% γ-PGA, 5% PBC and PBC+ γ-PGA (5%) on viability of *B. longum* NCIMB 8809 in simulated gastric juice (pH 2.0) (±SE). Protected and unprotected (control) cells were exposed to simulated gastric juice for 4hrs and viability was measured every hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3).

***B. breve* NCIMB 8807**

The protective effect of 5% γ-PGA, 5% PBC and PBC+γ-PGA (5%) was investigated when *B. breve* NCIMB 8807 was exposed to simulated gastric juice pH 2.0 for 4 hours in a microaerophilic condition (Figure 7.6). In the unprotected *B. breve* NCIMB 8807 cells, a loss in cell viability was recorded after one hour (5.05 Log CFU/ml). This was followed by total loss in cell viability by 2 hours.

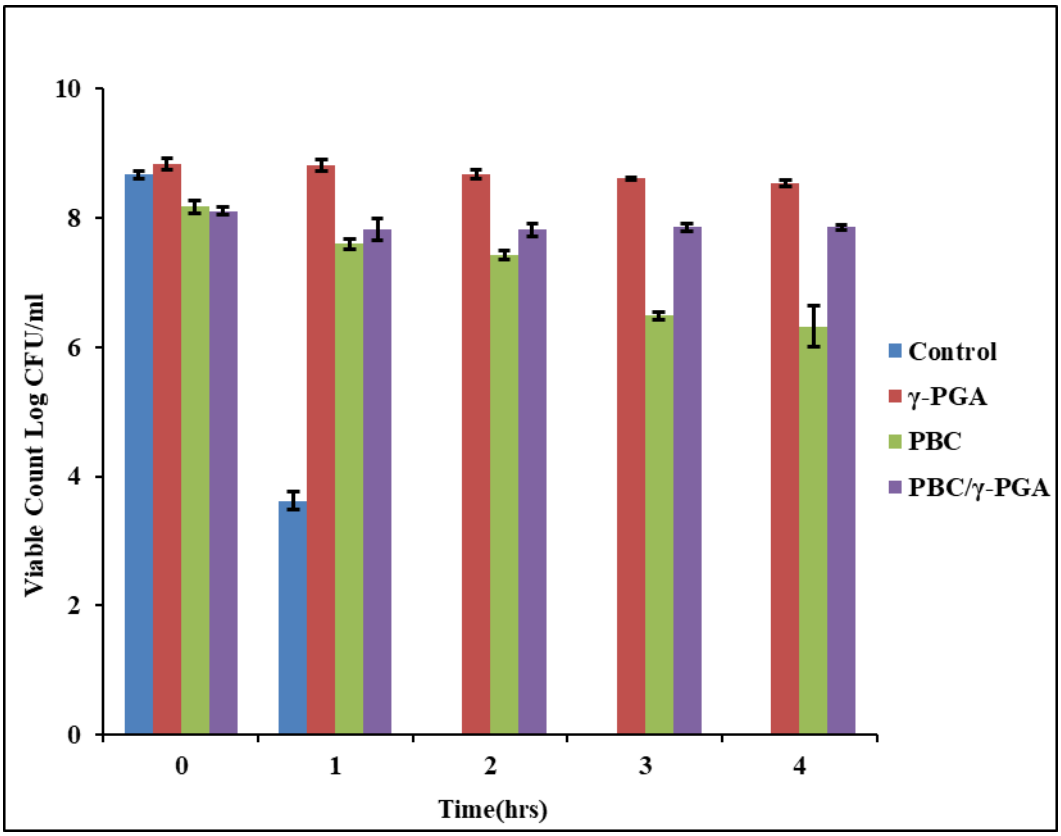


Figure 7.6: Protective effect of 5% γ-PGA, 5% PBC and PBC+ γ-PGA (5%) on viability of *B. breve* NCIMB 8807 in simulated gastric juice (pH 2.0) (±SE). Protected and unprotected (control) cells were exposed to simulated gastric juice for 4hrs and viability was measured every hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3)

When *B. breve* NCIMB 8807 was protected with 5% γ-PGA in simulated gastric juice, a total loss of 0.29 Log CFU/ml was recorded after 4 hours (Figure 7.6). The total number of cells at 0hr and at 4 hours were comparable and were not significantly different ($p>0.05$).

When *B. breve* NCIMB 8807 was protected with 5% PBC in simulated gastric juice, a total loss of 1.85 Log CFU/ml was recorded after 4 hours, statistical analysis revealed a significant difference between cell number at 0hr ad at 4hrs ($p<0.05$).

When *B. breve* NCIMB 8807 was protected with the combination of γ-PGA and PBC in simulated gastric juice, a total loss of 0.24 Log CFU/ml was recorded after 4 hours. Total number of viable cells at 0hr and at 4hrs were comparable and not significantly different ($p>0.05$).

***B. animalis* NCIMB 702716**

In the unprotected cells, a total loss of viability was recorded after 2 hours which is in contrast with the other strains where complete viability was lost after one hour. However, the total number of viable cells available after 1hr and 2hrs (3.76 Log CFU/ml and 3.67 Log CFU/ml respectively) were not enough to carry out any health benefits on the host.

When 5% γ-PGA was used to protect *B. animalis* NCIMB 702716 in simulated gastric juice, 0.54 Log CFU/ml reduction (figure 7.7) was recorded after 4 hours ($p<0.05$). When 5% PBC was used a 2 Log reduction in cell viability was recorded ($p<0.05$) while a total loss of 0.87 Log CFU/ml was recorded when a combination of γ-PGA and PBC was used to protect *B. animalis* NCIMB 702716 in simulated gastric juice ($p<0.05$).

7.3.2 Protection in Simulated Gastric Juice with 2.5% γ-PGA and 2.5% PBC

When a combination of γ-PGA and PBC (2.5% γ-PGA and 2.5% PBC) was used as a protective support for the 3 selected strains, the number of viable cells at 0hr and at 4hrs were

comparable and there was no significant difference in *B. longum* NCIMB 8809 and *B. breve* NCIMB 8807($p>0.05$), however, in *B. animalis* NCIMB 702716, there was a significant difference between viable cells at 0hr and 4hrs although the number of cells at 4hrs was in the recommended range (7.48 Log CFU/ml).

This led to investigating the protective effect of 2.5% γ-PGA and 2.5% PBC in simulated gastric juice for all 3 probiotic strains. Cell viability was measured every other hour (0, 2 and 4hrs).

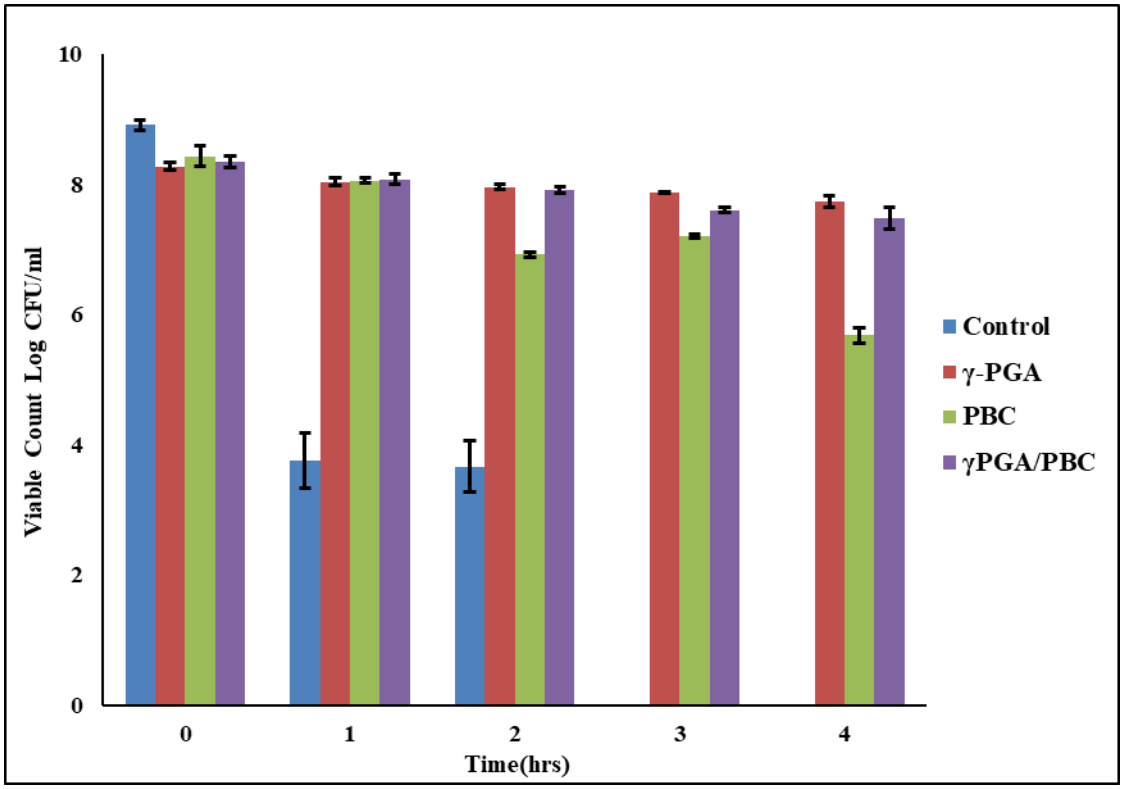


Figure 7.7: Protective effect of 5% γ-PGA, 5% PBC and PBC+ γ-PGA (5%) on viability of *B. animalis* NCIMB 702716 in simulated gastric juice (pH 2.0) (±SE). Protected and unprotected (control) cells were exposed to simulated gastric juice for 4hrs and viability was measured every hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3)

For the unprotected cells in all three strains, total loss in cell viability was recorded after 2 hours similar to previous results. When 2.5% γ-PGA was used as a protective support for *B.*

longum NCIMB 8809; a total of 0.23 Log CFU/ml was lost after 4hrs. The values of viable cells at 0hr and 4hrs were comparable and there was no significant difference ($p>0.05$). With 2.5% PBC; a loss in viability (3.17 Log CFU/ml) was recorded after 4hours ($p<0.05$) (figure 7.8).

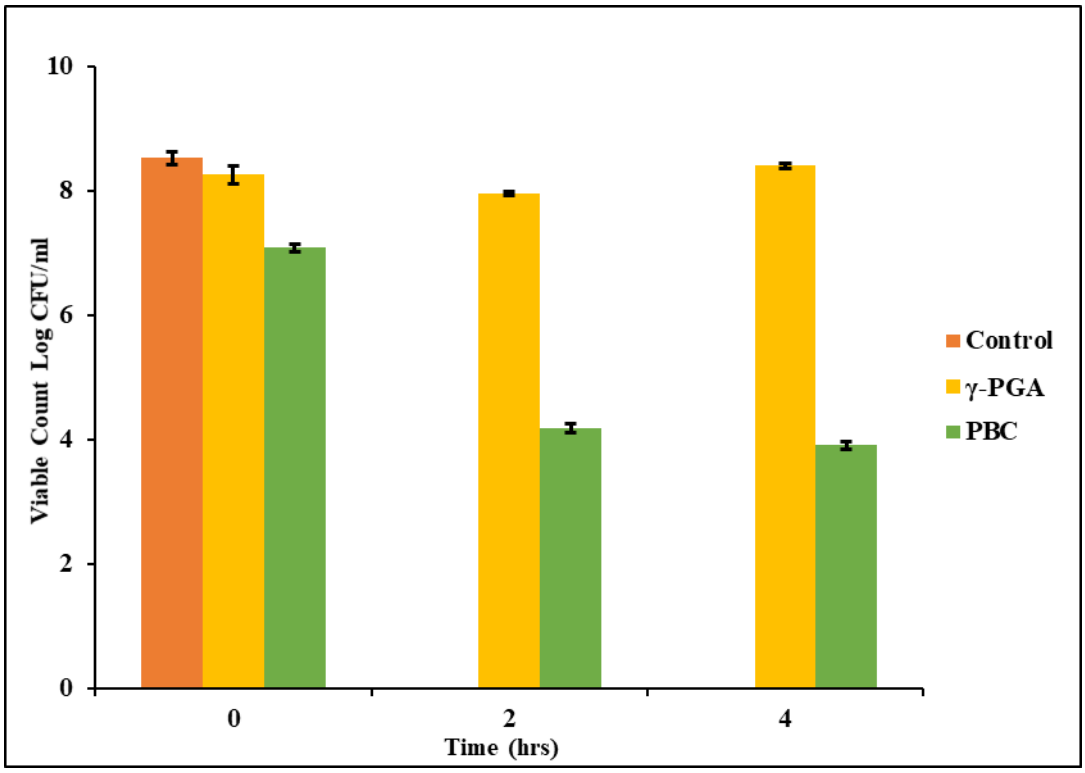


Figure 7.8: Protective effect of 2.5% γ-PGA and 2.5% PBC on viability of *B. longum* NCIMB 8809 in simulated gastric juice (pH 2.0) (±SE). Protected and unprotected (control) cells were exposed to simulated gastric juice for 4hrs and viability was measured every other hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3)

In *B. breve* NCIMB 8807, 0.10 Log CFU/ml reduction in viable cells was recorded after 4 hours when 2.5% γ-PGA was used (Figure 7.9). The mean number of viable cells at 0hr were comparable to the number of viable cells at 4hrs and not significantly different ($p>0.05$). When 2.5% PBC was used, 2.95 Log CFU/ml reduction in viable cells was recorded after 4hrs.

In *B. animalis* NCIMB 702716, when 2.5% γ-PGA was used, 0.10 Log CFU/ml reduction (Figure 7.10) in viable cells was recorded after 4hours ($p>0.05$) while a 4 Log loss was recorded when 2.5% PBC was used as a protective support for *B. animalis* NCIMB 702716 in simulated gastric juice

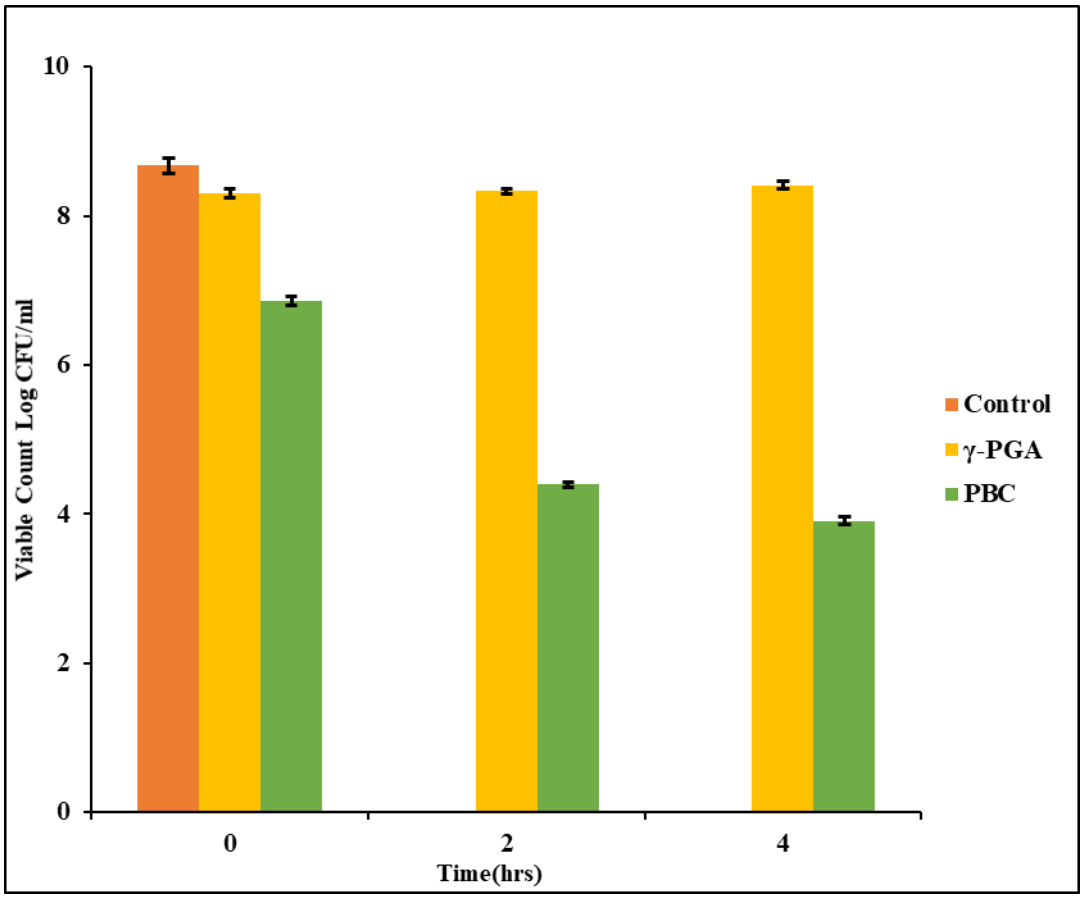


Figure 7.9: Protective effect of 2.5% γ-PGA and 2.5% PBC on viability of *B. breve* NCIMB 8807 in simulated gastric juice (pH 2.0) (±SE). Protected and unprotected (control) cells were exposed to simulated gastric juice for 4hrs and viability was measured every other hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3)

For the unprotected cells in the three strains investigated, the total loss in cell viability was recorded after 2 hours and the total number of viable cells after 4 hours in the 2.5% PBC

protected samples in all three probiotic strains were not enough to carry out any health benefit on the host.

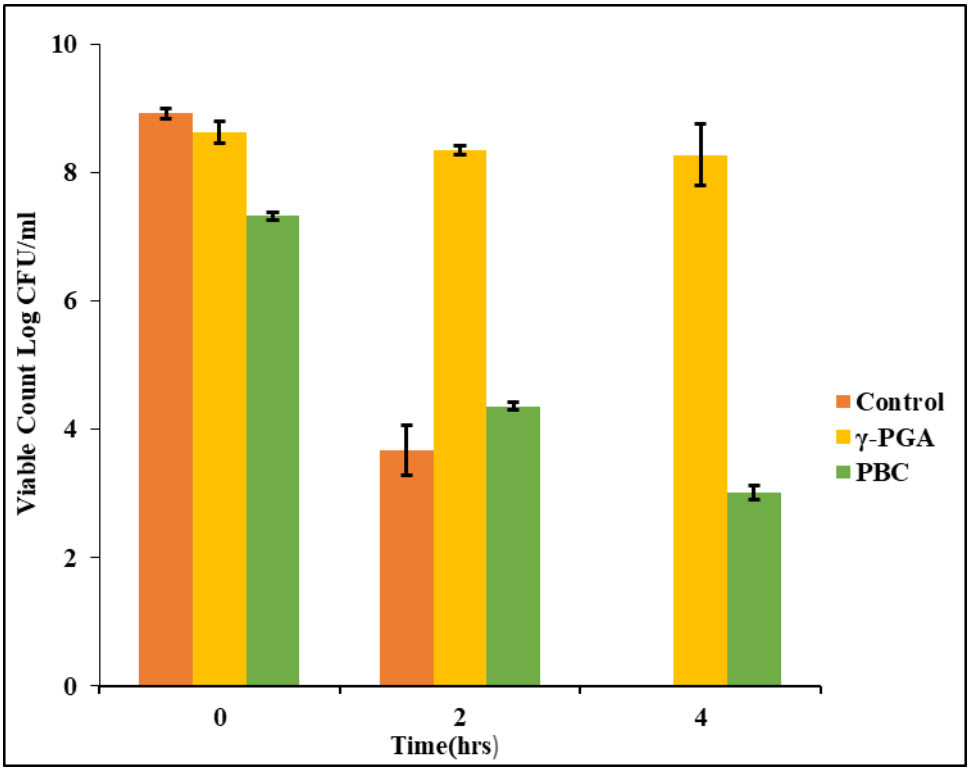


Figure 7.10: protective effect of 2.5% γ-PGA and 2.5% PBC on viability of *B. animalis* NCIMB 702716 in simulated gastric juice (pH 2.0) (±SE). Protected and unprotected (control) cells were exposed to simulated gastric juice for 4hrs and viability was measured every other hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3)

7.3.3 Protection in Simulated Intestinal Juice (SIJ)

The effect of exposing probiotic organisms to simulated intestinal juice was investigated using *B. breve* NCIMB 8807 protected with 5% γ-PGA, 5% PBC and PBC+ γ-PGA (5%). In the unprotected samples (control), there was no significant difference between cell viability at 0hr (8.84 Log CFU/ml) and at 3hrs (8.84 Log CFU/ml) ($p>0.05$). In γ-PGA protected

samples, cell viability at 0hr and at 3hrs were comparable and there was no significant difference between the ($p>0.05$). Likewise, in cells protected with PBC and γ-PGA+PBC, cell viabilities at 0hr and at 3hrs were comparable and there was no significant difference between them ($p>0.05$) (figure 7.11).

These results show that exposing probiotic organisms to SIJ has no detrimental effect on the survival of the strains during passage through the small intestine.

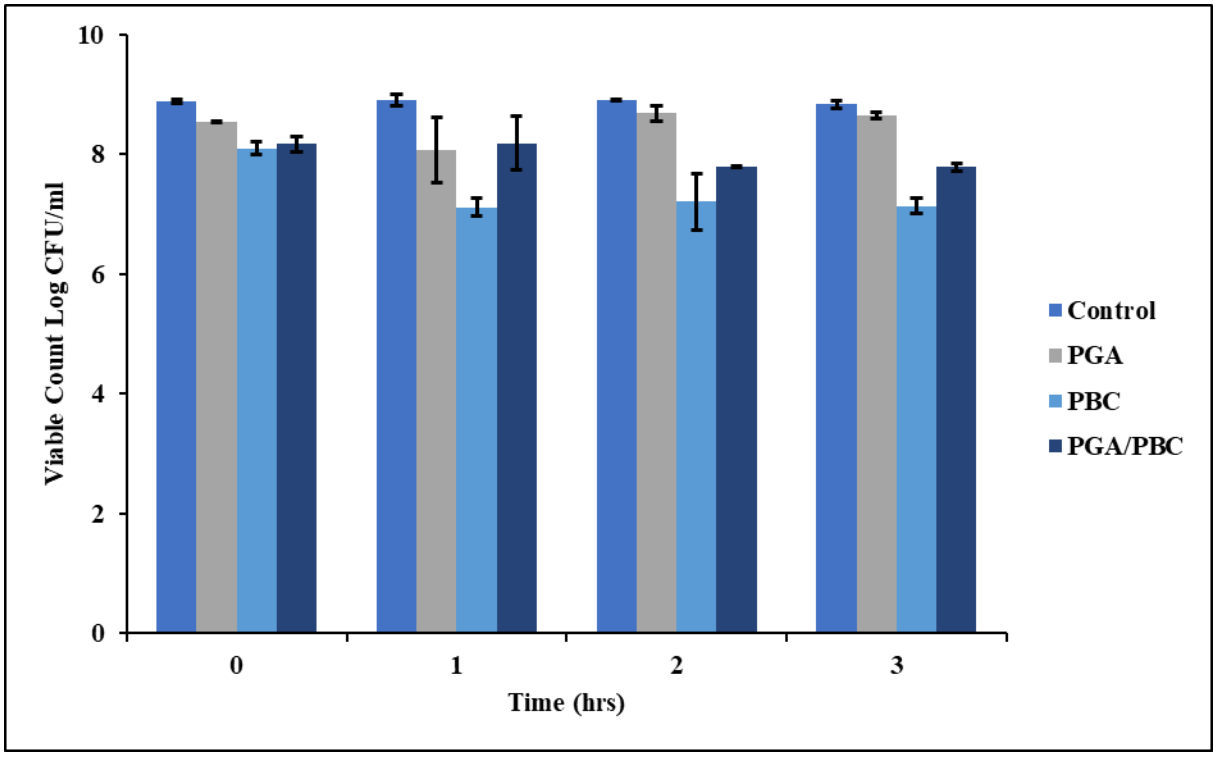


Figure 7.11: Protective effect of 5% γ-PGA, 5% PBC and PBC+ γ-PGA (5%) on viability of *B. breve* NCIMB 8807 in simulated intestinal juice (pH 6.0) (±SE). Protected and unprotected (control) cells were exposed to simulated intestinal juice for 3hrs and viability was measured every hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3)

7.3.4 Protection in Simulated Intestinal Juice and Bile Salts (SIJ+B)

Since bile salts are released into the small intestine to aid the digestion of fatty foods, it was important to investigate the effect of bile salts on the viability of probiotic organisms.

Simulated intestinal juice was modified by the addition of 1% (w/v) bile salts and the pH was adjusted to pH 6.0. The effect of protecting *B. breve* NCIMB 8807, *B. longum* NCIMB 8809 and *B. animalis* NCIMB 702716 with 5% γ-PGA, 5% PBC and 5% γ-PGA + PBC was investigated during exposure to bile salts in the presence of simulated intestinal juice (pH 6.0).

***B. longum* NCIMB 8809**

When *B. longum* NCIMB 8809 was exposed to bile salts in the presence of simulated intestinal juice for three hours, the control samples (unprotected cells) showed a reduction in viable cell count between 0hr and 2hrs (7.29 Log CFU/ml - 4.24 Log CFU/ml) and a total loss of viable cells was recorded after 3hrs (figure 7.12).

In γ-PGA protected cells, cell viability was maintained at 8 Log CFU/ml throughout the experiment and the cell viability at 0hr and 3hrs were comparable and there was no significant difference ($p>0.05$) (figure 7.12). When cells were protected with 5% PBC, there was a 2 Log reduction (8.23 Log CFU/ml - 6.91 Log CFU/ml) in cell viability after 3 hrs with a significant difference ($p<0.05$), however, the number of viable cells after 3hrs were enough to carry out health benefits on the host (figure 7.12).

When cells were protected with 5% γ-PGA+PBC; a slight increase in cell viability was observed between 0hr and 1hr (6.95 Log CFU/ml - 7.08 Log CFU/ml), cell viability remained at 7 Log CFU/ml between 1hr and 3 hrs. Cell viability at 0hr and 3hrs were comparable and there was no significant difference between the values ($p>0.05$).

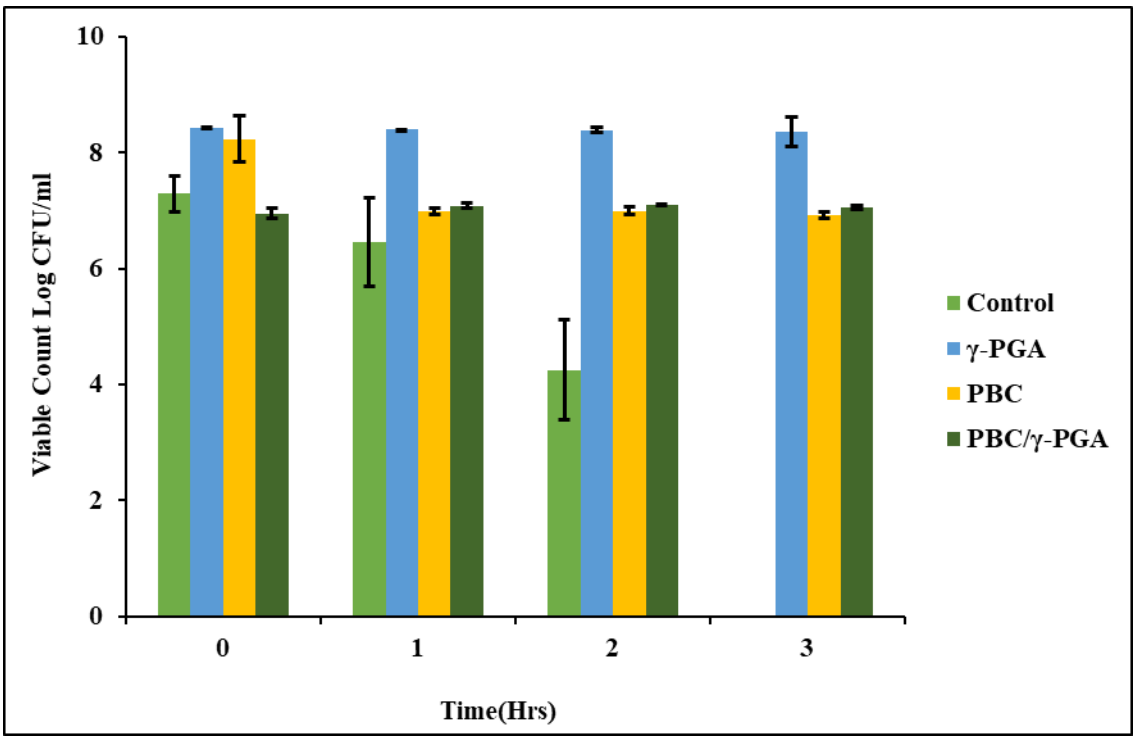


Figure 7.12: Protective effect of 5% γ-PGA, 5% PBC and PBC+ γ-PGA (5%) on viability of *B. longum* NCIMB 8809 in simulated intestinal juice + 1% bile salts (pH 6.0) (±SEM). Protected and unprotected (control) cells were exposed to simulated intestinal juice + 1% bile salts for 3hrs and viability was measured every hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3)

B. breve NCIMB 8807

In contrast to *B. longum* NCIMB 8809, unprotected *B. breve* NCIMB 8807 cells did not show total loss in viability throughout the duration of the experiment. However, a significant loss of viable cells was recorded (7.42 Log CFU/ml - 5.48 Log CFU/ml) during the 3 hours and there was a significant difference between these values ($p<0.05$) (figure 7.13).

In γ-PGA protected cells, a slight reduction in viability was observed after 3 hours (0.84 Log CFU/ml) and the cell viability at 0hr and at 3hrs were significantly different ($p<0.05$). In PBC protected cells, a one Log reduction in viable cells was recorded after 3hrs ($p<0.05$), and the viability was reduced from 7.07 Log CFU/ ml - 5.91 Log CFU/ml. In γ-PGA+PBC protected

cells, cell viability at 0hr and 3hrs were comparable and there was no significant difference between them ($p>0.05$). Cell viability was maintained at 7 Log CFU/ml during the period of the experiment. It can also be observed that viability slightly increased (7.65 Log CFU/ml - 7.83 Log CFU/ml) between 0hr and 3hrs, however, viablity was maintained at 7 Log CFU/ml during the experiment.

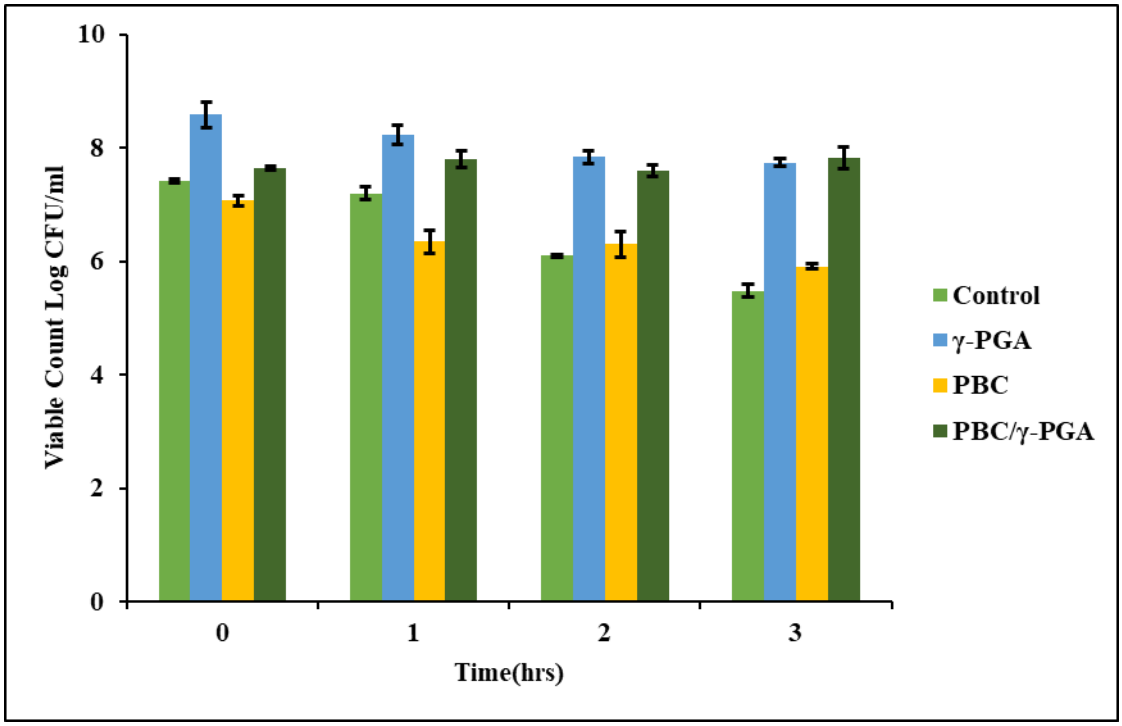


Figure 7.13: Protective effect of 5% γ-PGA, 5% PBC and PBC+ γ-PGA (5%) on viability of *B. breve* NCIMB 8807 in simulated intestinal juice + 1% bile salts (pH 6.0) (± SE). Protected and unprotected (control) cells were exposed to simulated intestinal juice + 1% bile salts for 3hrs and viability was measured every hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3)

B. animalis NCIMB 702716

Similar to *B. breve* NCIMB 8807, there was no complete loss of viable cells in unprotected *B. animalis* NCIMB 702716 cells, but, a reduction of 1.43 Log CFU/ml (7.15 Log CFU/ml - 5.73 Log CFU/ml) was recorded ($p<0.05$) after 3hrs (figure 7.14).

In γ-PGA protected cells, a slight reduction (0.13 Log CFU/ml) in viable cells was recorded. Viable cells at 0hr (8.08 Log CFU/ml) and at 3hrs (7.95 Log CFU/ml) were comparable and there was no significant difference between these values ($p>0.05$).

In PBC protected cells, a slight reduction (0.88 Log CFU/ml) in cell viability was recorded. Cell viability at 0hr and at 3hrs were significantly different ($p<0.05$), however, the number of viable cells at 3hrs (6.19 Log CFU/ml) were enough to carry out health benefits on the host.

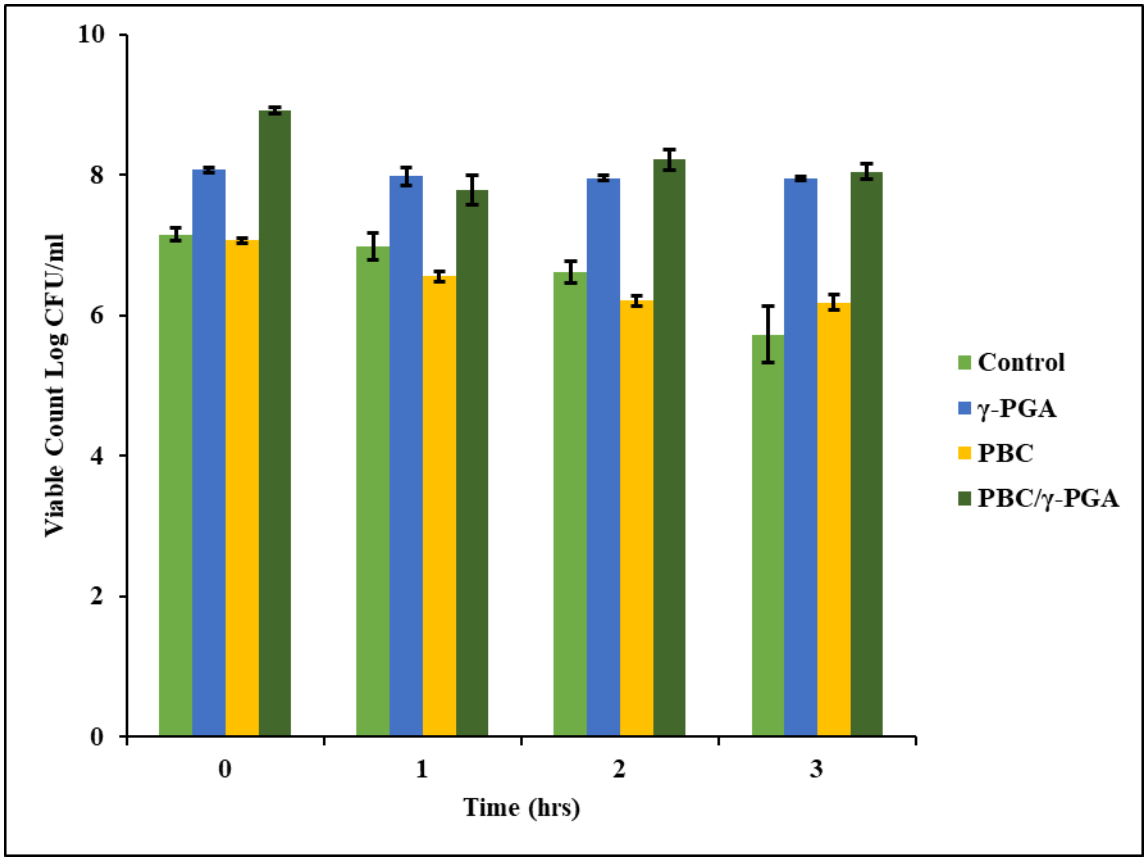


Figure 7.14: protective effect of 5% γ-PGA, 5% PBC and PBC+ γ-PGA (5%) on viability of *B. animalis* NCIMB 702716 in simulated intestinal juice + 1% bile salts (pH 6.0) (±SE). Protected and unprotected (control) cells were exposed to simulated intestinal juice + 1% bile salts for 3hrs and viability was measured every hour on BSM agar plates, plates were incubated anaerobically at 37°C for 48 hours (n=3)

In γ-PGA+PBC protected cells, there was a reduction in viable cell count after one hour (8.91 Log CFU/ml - 7.79 Log CFU/ml). This was followed by a slight increase in viable cell count

at 2hrs (7.79 Log CFU/ml - 8.22 Log CFU/ml), this could be due to attachement of cells to cellulose fibres and a reduction in viable cells was recorded at 3hrs (8.22 Log CFU/ml - 8.05 Log CFU/ml). The viable cells at 0hr and at 3hrs were comparable and there was no significant difference between these values ($p>0.05$).

8.0 DISCUSSION

8.1 Synopsis

This research was carried out to investigate the production of bacterial polymers (γ-PGA and bacterial cellulose), their protective effects on selected *Bifidobacterium* strains during freeze drying and exposure to simulated gastrointestinal conditions, and the antimicrobial properties of *Bifidobacterium* strains.

γ-PGA was produced by cultivating *B. subtilis* natto ATCC 15245 or *B. licheniformis* ATCC 9945a in GS or E media following incubation at 37°C, 150rpm for 96 hours. γ-PGA was recovered by centrifugation, ethanol precipitation, purification via dialysis and recovery of dry polymer via lyophilisation. The amount of γ-PGA produced by each bacterium in the different media was evaluated after recovery to determine which medium and strain is better for γ-PGA production for probiotic application. FT-IR and GPC were used to identify the polymer and determine the molecular weight respectively. Following characterization, *B subtilis* and GS medium were chosen as the preferred strain and cultivation medium respectively for further production for the probiotic application.

Bacterial cellulose (BC) was produced by cultivating *G. xylinus* ATCC 23770 in HS medium or a modified HS medium (MHS) statically at 30°C for 14 days. BC was produced as pellicles in the air-liquid interface of the culture medium, BC pellicles were removed and purified by hot alkali and water treatment. The amount of wet BC pellicles from each media was determined, then BC pellicles were freeze-dried followed by determination of the dry weight. FT-IR, SEM and XRD were used for identification, investigating the external morphology and fibre network and investigating the crystallinity of BC samples. BCs obtained from both media were similar, thus MHS medium was selected for further production and milling and for probiotic application due to the higher polymer yield compared to HS medium.

The cryoprotective effect of wet BC sheets, γ-PGA or powdered BC (PBC) on six *Bifidobacterium* strains (*B. longum* NCIMB 8809, *B. breve* NCIMB 8807, *B. animalis* NCIMB 702716, *B. bifidum* NCIMB 702715, *B. infantis* NCIMB 702255 and *B. adolescentis* NCIMB 702229) were investigated and compared with sucrose and skimmed milk powder (SMP). The probiotic strains were grown in TPY medium, cells were collected by centrifugation, mixed with sterile cut cubes of BC or suspended in 5% γ-PGA (w/v), 5% PBC (w/v), 5% sucrose (w/v) or 5% SMP (w/v) and the number of viable cells after freeze drying was determined.

Antimicrobial activity is one of the unique properties of probiotic bacteria and this has been reported to be strain specific (Shokryazdan *et al*., 2014). Thus, the antimicrobial properties of the six *Bifidobacterium* strains listed above against selected pathogens (*E. coli* W1485-K12 W-T, *S. typhimurium* WLV 73 Cardiff Collection, *S. aureus* NCIMB 6571 and *L. innocua* NCTC 11288) were investigated during this study using the well diffusion assay technique. Cell free culture supernatants obtained from each probiotic strain was grouped into two, treated (pH was adjusted to pH 7.0) and untreated. The antimicrobial activities of each group were then investigated in the non-concentrated and concentrated state. Production of organic acids (lactic and acetic) and change in pH of culture medium was investigated during this study, since previous studies have shown that there is a link between organic acid production, pH reduction and antimicrobial activities (Likotrafiti *et al*., 2013; Trejo *et al*., 2006).

One of the major challenges of delivering probiotic bacteria to the target site alive and in the adequate amount (> 7 log CFU/g) is exposure to gastrointestinal conditions (Patrignani *et al*., 2017). This study investigated the survival of probiotic bacteria (*B. longum* NCIMB 8809, *B, breve* NCIMB 8807 and *B. animalis* NCIMB 702716) protected with γ-PGA, PBC or γ-

PGA+PBC (5% w/v) during exposure to simulated gastric juice, simulated intestinal juice and during exposure to simulated intestinal juice plus 1% bile salts.

8.2 Growth and Production of γ-PGA in GS Medium and Medium E by *Bacillus* Species

Production of γ-PGA was investigated in *B. subtilis* natto ATCC 15245 and *B. licheniformis* ATCC 9945a at 37°C for 96 hours in GS and E media. Both strains have been used previously to investigate the production of γ-PGA and they have been reported to produce γ-PGA with different physical and chemical properties depending on medium components and culture conditions (Kedia *et al*., 2010).

It was important to investigate bacterial growth during cultivation since medium composition has been reported to support bacterial growth which will generate enough precursors such as α-ketoglutaric acid and L-glutamine for γ-PGA synthesis and have an overall influence on the polymer yield (Luo *et al*., 2016).

γ-PGA concentration has been reported to reach its maximum during the late exponential phase and early stationary phase (Bajaj and Singhal, 2011). When both strains were grown in GS medium, both strains attained the exponential phase at 24 hours and the stationary phase by 48 hours (Figures 5.1a and 5.2a). However, when both strains were grown in medium E, it took a longer time to attain the stationary phase (figures 5.1b and 5.2b), this is possibly due to the presence of high glycerol concentration (80g/l). This finding is in accordance with the observation of Wu *et al*. (2010), who demonstrated that high glycerol concentration (≈80g/l) results in inhibition of cell growth in *B. subtilis* NX-2.

During cultivation, viscosity of GS cultivation medium was obvious after 24 hours and increased until the end of production, while viscosity was observed in medium E after 48

hours. Viscosity in both media is an indication of the formation of γ-PGA and it has been linked with the stationary phase of growth. Shih *et al*. (2002), reported that the relative viscosity of the medium increased with the incubation time when γ-PGA production was optimised using statistical experimental methods, they observed that the relative viscosity of the medium increased with the incubation time and reached its maximum after 96 hours. Other reports (Kumar and Pal, 2015; Mitsui *et al*., 2011) have shown that γ-PGA formation occurs during the early exponential phase and reaches maximum concentration during the late exponential phase of growth and early stationary phase.

γ-PGA synthesis is dependent on the *pgsB* gene, which encodes the γ-PGA synthase complex (pgsBCA) the sole machinery of γ-PGA production with a catalytic site for γ-PGA polymerization (Ashiuchi *et al*., 2001; Candela and Fouet, 2006). According to Kimura *et al*. (2009), *pgsB* is expressed in the early stationary phase of growth when *B. subtilis* natto was used for production of γ-PGA. This clearly explains why γ-PGA production takes place during the stationary phase of growth.

The effect of the vitamin solution present in GS medium was noticeable for *B. subtilis* natto ATCC 15245 with a higher cell count when grown in the presence of vitamin solution (GS medium) than when grown without vitamin solution (medium E) as presented in figure 5.1. This is similar to a previous study where it was reported that *B. subtilis* natto is fastidious in nature, hence grows faster with the addition of vitamins (Kedia *et al*., 2010).

During cultivation, as γ-PGA production increases in culture medium, the medium becomes viscous which affects volumetric oxygen transfer, resulting in limited oxygen availability and consequently lowering γ-PGA production. Hence, controlling cultivation conditions such as aeration, agitation and pH becomes important for optimised γ-PGA production, since nutrient transfer and the distribution of air and oxygen are dependent on agitation, while bulk mixing

of the fermentation culture and culture oxygenation are determined by aeration (Bajaj and Singhal, 2011).

However, this is difficult to achieve when cultivation is performed in shake flasks as was the case during this study (agitation was maintained at 150rpm). A maximum cell count of 9.07 Log CFU/ml was recorded in both strains, in both media and none of the strains reached a count of 10 Log CFU/ml. This could be due to cultivation in an environment where pH and aeration could not be controlled. Cell counts of 10 Log CFU/ml have been reported previously by Kedia *et al*. (2010), when *B. subtilis* natto was grown in GS medium in the fermenter, where agitation was increased from 150rpm - 800rpm and air flow rate was increased to 5 L/min.

The effect of cultivation in a non-controlled environment was also noticeable with the change in pH in both media (Table 5.1); an initial pH of 6.80 in GS medium was reduced to pH 6.16 during the exponential phase and remained constant until the early stationary phase. In medium E, a reduction in pH was observed throughout the cultivation period (Table 5.1). This is similar to the observations reported by Kumar and Pal (2015) during the investigation of γ-PGA production from *B. licheniformis* NCIM 2324, where carbon source, bacterial growth, pH, dissolved oxygen, and γ-PGA and glutamic acid concentrations were monitored with time. A reduction in pH from 6.5 - 4.8 was recorded as γ-PGA concentration reached 36g/l. The reduction in pH is believed to be due to the accumulation of acid in the culture medium. A reduction in pH from pH 7.0 to < pH 6.0 followed by a slight increase in pH to ≈ pH 7.50 was also observed by Kongklom *et al*. (2015) during the production of γ-PGA in shake flasks. It was observed that increase in pH correlates with reduction in glucose concentration and suggested that monitoring pH can be used to enhance γ-PGA productivity.

It is clear that pH, aeration and agitation are important factors influencing bacterial growth and γ-PGA production. To effectively optimise γ-PGA production, cultivation in a pH controlled, and high aerated environment is crucial.

Following extraction and recovery of polymer, obtained polymer was weighed to determine and calculate the total yield in g/l. Total polymer yield in both media by the two strains were compared, represented in figure 5.3. Polymer yield was strain and medium dependent, when *B. subtilis* natto ATCC 15245 was grown in GS and E media, an average yield of 14.11±0.09g/l and 6.03±0.06g/l respectively were obtained. When *B. licheniformis* ATCC 9945a was grown in GS and E media, an average yield of 11.05±0.09g/l and 10.27±0.15g/l respectively were recorded, there was a significant difference between these values ($p>0.05$) but the difference in yield is marginal (0.78g/l) compared with a difference of 8.08g/l in *B. subtilis* natto ATCC 15245 in GS and E media. This further proves that polymer yield is dependent on strain, growth condition and medium components according to the reports of Zeng *et al*. (2016) where it was reported that apart from screening specific strains, regulating medium components is an effective method for improving polymer yield.

The fact that slightly higher yields were obtained using GS medium when compared with medium E in both strains (see figure 5.3) is similar to results obtained by Shih *et al*. (2006) using sucrose as a preferred carbon source. Whilst they investigated seven different carbon sources to produce γ-PGA using *B. subtilis* ZJU-7, it was reported that sucrose as a carbon source gave the highest rate of γ-PGA productivity.

The variation in polymer yield obtained from both media could also be due to medium components since a high concentration of glycerol has been linked with reduction in cell growth and overall γ-PGA production, Wu *et al*. (2010), reported inhibition of cell growth with glycerol concentration as high as 80g/l which is used in the preparation of medium E.

The low yield obtained when *B. subtilis* natto ATCC 15245 was grown in medium E (6.03±0.06g/l) could be a result of degradation of produced γ-PGA, since past findings have shown that as γ-PGA is produced extracellularly, it can be exposed to degradation enzymes (GGT and YwtD) responsible for γ-PGA degradation, Mitsui *et al*. (2011) observed the production of these enzymes in *B. subtilis* natto and suggested they might have a negative effect on γ-PGA production.

Another possible explanation could be due to the fastidious nature and nutritional requirements of *B. subtilis* natto reported by Kedia *et al*. (2010). Our results agree with the report of Kedia *et al*. (2010) where maximum γ-PGA yield was recorded when *B. subtilis* natto was grown in GS medium, and a yield of less than 1g/l was recorded in medium E. In addition, it was also discovered that addition of vitamins to the culture medium for *B. subtilis* natto ATCC 15245 is not essential since the organism produced γ-PGA in the absence of vitamins in medium E, although the polymer yield was higher in GS medium which is vitamin supplemented. The production of γ-PGA in *B. subtilis* 2063 was studied by Bhunia *et al*. (2012), although there was no report of supplementing the culture medium with vitamins. However, γ-PGA was reportedly produced in a high concentration (21.42g/l).

These studies clearly point to the fact that nutritional requirements of these strains vary and that production of γ-PGA by *B. subtilis* natto ATCC 15245 is not dependent on supplementing the culture medium with vitamins. However, supplementing with vitamins might enhance γ-PGA production in these strains.

Overall, both strains used for this study, produced γ-PGA in varying concentrations in the presence of GS and E media. It is also evident that production of γ-PGA is dependent on the bacterial strain, medium composition and growth condition, as has been found previously (Ogunleye, 2012).

After the extraction and recovery processes, polymer obtained from GS medium was whitish in colour while the polymer obtained from medium E was brownish in colour. This is probably due to the presence of iron (III) chloride hexahydrate in the medium (Table 4.3), it is important to note that it was white in colour during the precipitation step, but it changed to brown colour after exposure to oxygen. The colour of γ-PGA could play important role in its application; white γ-PGA can be employed in food, medical and cosmetic application while the brownish coloured γ-PGA can be used in its natural form for waste water treatment.

Both strains under investigation produced γ-PGA as confirmed by FT-IR analysis, the spectra (figures 5.4 and 5.5) obtained from the FT-IR analyses of all samples were similar and comparable to the commercial sample. The presence of hydroxyl, carboxyl and amide groups in the produced γ-PGAs is confirmed by the absorption peaks at the specific wavelengths in the FT-IR spectra of the γ-PGAs produced in this study. These results are consistent with the studies of Khalil *et al*. (2016) who analysed the structural characteristics of the polymer obtained from the fermentation of *B. subtilis* natto using FT-IR. The presence of hydroxyl, carbonyl, amide and carboxyl groups was confirmed by the presence of the different absorption peaks at specific wavelengths in the spectra of γ-PGAs obtained in this study.

Molecular weight is one of the important properties of γ-PGA as this is one of the determining factors of its application and reports have shown that determination of molecular weight of γ-PGA is important to understand its functions (Zeng *et al*., 2016). The number of glutamic acid residues the bacteria can polymerise before transporting it outside the cell, the presence of extracellular depolymerase activity and the availability of nutrients in the cultivation medium are factors that can affect molecular weight of γ-PGA (Kimura *et al*., 2004). Other factors that can affect molecular weight of γ-PGA include medium components and bacterial strain. Sung and co-workers (2005) reported that in *B. subtilis* chungkookjang,

altering the NaCl concentration in a particular range (5g/l - 100g/l) could be used to control the molecular weight of γ-PGA, whilst Buescher and Margaritis (2007) reported that under the same cultivation conditions, *Bacillus* sp. RKY3 produced γ-PGA of 10 - 50 k Da while *B. subtilis* chungkookjang formed γ-PGA of over 10,000 k Da.

Shih and Van (2001) reported that molecular weight and dispersity are crucial properties of γ-PGA and that γ-PGA produced by *Bacillus* species are generally of high molecular weight, with values ranging from 10^5 - 10^6 Da and dispersity of between 2 and 5. As mentioned previously, different applications require γ-PGA of varying molecular weight. Shi *et al*. (2003) reported that the antifreeze property of γ-PGA reduces with increase in molecular weight. γ-PGA with molecular weight of about >2000 k Da was reported to stimulate immune response against viral antigens in rabbits (Buescher and Margaritis, 2007), whilst Inbaraj *et al*. (2006a) reported that γ-PGA with molecular weight 990 k Da is effective for waste water treatment. Hence, there was a need to determine the molecular weight of γ-PGAs obtained during this study and this was done using GPC. *B. subtilis* natto ATCC 15245 produced γ-PGA with lower molecular weights (Table 5.2) when grown in GS and E media ($1.28x10^6$ Da and $1.10x10^6$ Da respectively), compared with *B. licheniformis* ATCC 9945a in GS medium and medium E ($1.42x10^6$ Da and $1.62x10^6$ Da respectively). The molecular weight analysis from this study is similar to the reports of Buescher and Margaritis (2007) and Zeng *et al*., (2016) that molecular weight of γ-PGA is strain and medium composition dependent.

B. subtilis natto ATCC 15245 produced γ-PGA with a molecular weight of $1.28x10^6$ Da in GS medium while γ-PGA with molecular weight of $1.10x10^6$ Da was released when grown in medium E, this could be due to the presence of glycerol (80g/l) in medium E which is absent in GS medium as reported by Wu *et al*. (2010) that high concentrations of glycerol in culture

medium could result in reduction of molecular weight. However, a higher molecular weight ($1.62x10^6$ Da) γ-PGA was obtained when *B. licheniformis* ATCC 9945a was grown in medium E compared to molecular weight $1.42x10^6$ Da obtained in GS medium. This further shows that molecular weight is strain specific and medium dependent.

Finally, low molecular weight γ-PGA has been reported to have better antifreeze properties (Shih *et al*., 2003), and a high molecular weight γ-PGA was obtained during this study, this can be reduced by hydrolysis in aqueous solution. The decomposition temperature of Na-γ-PGA has been reported to be 340°C (Ho *et al*., 2006), hence, sterilising at 110°C for 30 minutes will not have a detrimental effect on the physical and chemical properties of γ-PGA which is important because in addition to reducing the molecular weight of the γ-PGA, unwanted residual bacteria that might interrupt subsequent viable cell count results for probiotic experiments will be eliminated.

8.3 Scale-Up Production of γ-PGA by *B. subtilis* Natto ATCC15245

Following production in shake flasks and characterization of γ-PGA, GS medium was selected as the preferred production medium, while *B. subtilis* natto ATCC 15245 was selected as best bacteria for the following reasons:

- Previous studies from our lab (results not shown) revealed that Na-γ-PGA was produced when *B. subtilis* natto ATCC 15245 and *B. licheniformis* ATCC 9945a were grown in GS medium. Na-γ-PGA has been reported to have very good antifreeze properties according to the reports of Bhat *et al*. (2013) and can be used as a cryoprotectant for probiotic bacteria.
- Na-γ-PGA has been reported to have a weaker taste compared with other low molecular weight cryoprotectants, hence, it can be added in large quantity without a serious effect on the taste of the food (Ho *et al*., 2006).

- *B. licheniformis* ATCC 9945a produced γ-PGA with a higher molecular weight compared with *B. subtilis* natto ATCC 15245, this makes *B. subtilis* more desirable as lower molecular weight γ-PGA has better antifreeze properties (Shih *et al*., 2003).
- Although the molecular weight of γ-PGA form *B. subtilis* natto ATCC 15245 is higher than that obtained from past studies, it can be reduced by hydrolysis.
- Finally, the highest γ-PGA yield was recorded when *B. subtilis* natto ATCC 15245 was grown in GS medium.

It was important to scale up production of γ-PGA from the 250 ml shake flasks where cultivation conditions could not be monitored to a 5l fermentation vessel where cultivation conditions were closely monitored and ultimately increasing polymer yield.

When *B. subtilis* natto ATCC 15245 was grown in GS medium in a fermenter, a maximum cell count of 8.57 Log CFU/ml was achieved after 72 hours. This compares with growth in shake flasks, where a maximum cell count of 8.60 Log CFU/ml was recorded after 24 hours with the same strain. This result contradicts past findings in our lab (results not shown) where cell count reached a maximum of 10 Log CFU/ml, however, maximum cell count was achieved in both studies around the same time (72 hours).

An average γ-PGA yield of 24.15 g/l was recorded during fermentation in a fermenter; this is significantly higher than the yield obtained (14.11 g/l) when *B. subtilis* natto ATCC 15245 was grown in GS medium using shake flasks. This result is comparable to results obtained in the study of Kedia *et al*. (2010) where a yield of ≈28g/l was reported during γ-PGA production using a fermenter. The significantly higher yield obtained in this study can be linked to the optimized growth conditions and not cell growth as a higher maximum cell count (8.60 Log CFU/ml) was recorded during fermentation in shake flasks.

FT-IR analysis revealed obtained polymer is γ-PGA, the spectrum obtained from the fermenter γ-PGA was comparable with spectrum obtained from commercial γ-PGA revealing all expected absorption peaks although the γ-PGA from the fermenter showed an additional peak at 1631 cm^{-1} which corresponds to amide 1 N-H bending (figure 5.7). Molecular weight of γ-PGA obtained from the fermenter was not investigated, since the molecular weight of γ-PGA obtained from fermentation of *B. subtilis* natto ATCC 15245 in GS medium using shake flasks in our pilot studies had already been determined.

8.4 Growth and Production of BC in Different Media by *Gluconacetobacter xylinus* ATCC 23770

G. xylinus is mostly studied for production of BC in different media with different carbon sources such as glycerol (Jung *et al*., 2010), fructose (Chao *et al*., 2001), mannitol (Sheykhnazari *et al*., 2011), fructose supplemented with corn steep liquor (CSL) (Bae *et al*., 2004), coconut water (Phisalaphong and Jatupaiboon, 2008) or glucose (Gupta *et al*., 2016); glucose supplemented with additives such as methanol, ethylene-glycol, glycerol (Lu *et al*., 2011). Glucose is mostly used as carbon source, probably due to its easy availability (Kuo *et al*., 2016).

Jagannath and co-workers (2008) studied the effect of excluding citric acid from the HS medium. Although the pH was adjusted to 4.2 with acetic acid, BC was reportedly produced in the absence of citric acid at pH 4.2. Their study led to the modification of HS medium for our study, with pH being adjusted to 6.0 with glacial acetic acid for this study. *G. xylinus* has been reported to convert glucose to gluconic acid during fermentation, which results in reduction in pH, cell growth and ultimately reduction in BC formation (Hwang *et al*., 1999), hence, the reason behind adjusting pH to 6.0 in this study.

When *G. xylinus* ATCC 23770 was grown statically at 30°C for 14 days in HS medium and MHS medium, both media were distinctly turbid with no BC formation observed on day 2 and a one Log increase in cell count (Figure 6.1 and 6.2). A film of BC was observed on both media surfaces on day 4 with a reduction in cell number from 6.28 Log CFU/ml to 6.16 Log CFU/ml in HS medium, this is probably due to attachment of cells to formed BC pellicles while MHS medium showed an increase in cell number from 6.17 Log CFU/ml to 6.29 Log CFU/ml.

Exponential phase was fully achieved on day 6 in both media (6.33 Log CFU/ml in HS medium and 6.50 Log CFU/ml in MHS medium where maximum number of viable cells was recorded). At this stage, BC pellicles were thicker and the low cell count is probably due to the attachment of bacterial cells to cellulose pellicles. This result is in line with past findings that cellulose production begins during the growth phase and that *G. xylinus* demonstrates slow growth in static conditions, while rapid increase in cell growth is associated with aerated and agitated conditions (Hwang *et al*., 1999; Jagannath *et al*., 2008). Some limiting factors have been reported under agitated conditions, such as sensitivity of *Gluconacetobacter* strains to shear forces (Son *et al*., 2003) and production of non-cellulose producing mutants which will result in low BC yield (Ul-Islam *et al*., 2015). The stationary phase was difficult to notice in HS medium (figure 6.1), although it was observed in MHS medium (figure 6.2) after day 6 followed by decline in cell number. This is not surprising as cell growth has been reported to be slower under static conditions (Jagannath *et al*., 2008). Hwang and co-workers (1999) reported that the stationary phase was achieved between 35-45 hours during the production of BC from *A. xylinum* BRC5 under aerated and agitated conditions. Lu *et al*. (2011), demonstrated that during the production of BC by *G. xylinus* 186 with optimised alcohol concentrations, BC production was obvious around day 2 - 4 of fermentation, which

is in accord with our results, a low cell number was recorded at 24 hours followed by an increase and the stationary phase began around day 3 - 4 of fermentation.

The pH of both media was monitored during the fermentation process, since reports have shown that BC producing *Gluconacetobacter* strains can actively convert glucose to keto-gluconic acid during fermentation by a membrane-bound dehydrogenase, causing a reduction in pH, thus having an overall effect on BC production (Jung *et al*., 2010; Zhang *et al*., 2016). Klemm *et al*. (2001) also reported that the production of gluconic acid is important for BC formation by initially lowering the environmental pH followed by the internal pH which then results in stabilization and or activation of key enzymes required for BC synthesis.

In this study, the initial pH of both media was 6.0. On day 2, a reduction in pH to ≤ pH 5.88 was observed in both media (Table 6.1). This result is similar to the report of Hwang *et al*. (1999) where the accumulation phase of gluconic acid was reported to be around 8 - 21 hours in agitated conditions.

A slight increase in pH was observed on day 12 in HS medium (pH 4.72 – pH 4.85) and on day 10 in MHS medium (pH 4.78 – pH 4.81). The slight increase in pH could be due to oxidation of produced gluconic acid by *G. xylinus*. Rangaswamy *et al*. (2015) reported that once glucose has been completely oxidized in the production media, the bacteria begins to metabolize released gluconic acid which then results in a gradual increase in the pH of the culture medium.

Lu *et al*. (2011) reported that the production of BC is a more complex process and that reduction in pH was not the only factor affecting BC production as pH either increased, reduced or stayed the same in their study with alcohol supplementation using mannitol, glycerol, methanol, ethylene glycol, n-propanol or n-butanol.

BC pellicles were observed on the culture medium as a thin film layer around the fourth day of fermentation, the pellicles became thicker in some flasks while in some flasks, pellicles sank to the bottom of the flask and then new cellulose pellicles were formed on the surface of the medium. Once harvested on day 14, an average thickness ≈10mm was recorded in BC pellicles from HS medium while an average thickness of ≈14mm was recorded in BC pellicles from MHS medium. This is in accord with the report of Auta *et al*. (2017) where an average thickness of 12mm was reported in BC pellicles obtained from fermentation of *G. xylinus* 639 in HS medium.

It was important to investigate the weight of the BC obtained from both media to select which media to use for further work. After purification of BC pellicles, wet and dry weights of BC pellicles were determined. When *G. xylinus* ATCC 23770 was grown in HS medium, a yield of 31.92±1.78 g/l was recorded while a yield of 78.88±4.28 g/l (Table 6.2) was recorded when *G. xylinus* ATCC 23770 was grown in MHS medium which is similar to the report of Auta *et al*. (2017) where a wet weight of 90.48±12.14 g/l was recorded.

Following freeze drying and determination of dry weight, dry weights of 0.78±0.04 g/l and 1.37±0.07 g/l were recorded in HS and MHS media respectively. The dry weight of BC obtained from HS medium in this study corresponds to results obtained from past studies during the investigation of BC production in a static environment. An average dry weight of 1.4±0.09 g/l was reported when *G. xylinus* was grown statically in HS medium (Auta *et al*., 2017), whilst Kuo *et al*. (2016) reported a dry weight of 1.23g/l and Son *et al*. (2003) reported a dry weight of 1.58 g/l when *G. xylinus* was grown in HS medium. However, a dry weight of 0.85g/l was reported by Lu *et al*. (2011), when the stimulatory effect of 6 alcohols was investigated and compared with medium with no alcohol supplement. Addition of

alcohol as supplements improved BC production, a yield of ≈13.36 g/l was recorded compared with non-supplemented medium with a yield of 0.85g/l.

To the best of our knowledge, the work done by Jagannath and co-workers (2008), where citric acid was excluded from the components of HS medium to produce BC is the only report to date that explains the effect of excluding citric acid from the medium. Their report only confirms BC can be produced from HS medium in the absence of citric acid and no report of BC yield. In this study, we have explored production of BC in MHS medium at pH 6.0, since reports have shown that this is the optimum pH for BC production (Rangaswamy *et al*., 2015). Reduction in pH is detrimental to BC production, which is to be expected, since glucose is oxidized to gluconic acid during production of BC (Zhang *et al*., 2016).

The dry weight of BC obtained from MHS medium (1.37g/l) is over 1.5-fold greater than BC obtained from HS medium (0.78g/l), this further confirms the work of Jagannath and co-workers (2008) that BC can be produced in the absence of citric acid. Our results also show that BC can be produced in the absence of citric acid at pH 6.0 and an improved yield of BC was recorded. It is important to note that the presence of an organic acid is essential, in the report of Jagannath *et al*. (2008), the pH was adjusted to 4.2 using acetic acid and in our study pH was adjusted to 6.0 using acetic acid in both media.

The low yield obtained during this study can be linked to inability to control conditions such as pH, aeration and agitation. Controlling pH is an important aspect of BC production but, this is a difficult feat to achieve by autoregulation using a pH sensor, this is because BC attaches to the sensor during fermentation leading to inaccurate pH readings (Kuo *et al*., 2016). Hwang and co-workers (1999) reported an improved BC production when the dissolved oxygen was controlled in a jar fermenter with glucose as sole carbon source. A yield of 10g/l of BC was obtained from 40g/l glucose.

In an agitated environment, mixing of the culture broth is difficult due to its high viscosity. Kouda *et al*. (1997) reported that with specialized impellers, such as the Maxblend and gate with turbine impellers, mixing of the culture broth was improved because of the better oxygen transfer capacity of the specialized impellers, which resulted in improved BC production.

BC is highly hydrophilic and has a high water holding capacity (WHC). It can hold water almost 100 times its dry weight, the WHC of wet BC reduces drastically after air-drying, since porosity of BC can be affected depending on the drying method, whilst freeze drying has been described as the best method of drying for preservation of its porosity (Andrade *et al*., 2010). Our study is in accord with this report, which is evidenced with the difference in the weight of BC before freeze drying (31.92 g/l, 78.88 g/l) and weight after freeze drying (0.78 g/l and 1.37 g/l) in HS and MHS medium respectively.

FT-IR, SEM and XRD were used for the identification and characterization of BC obtained from both media. FT-IR identified obtained polymer as cellulose and the spectra obtained from the analyses of BC from both media were similar and comparable with FT-IR spectra of commercial cellulose (Figure 6.4). A shift in the FT-IR of BCs from this study was also observed between 1620 cm^{-1} -1422 cm^{-1} due to washing with NaOH. The FT-IR spectra obtained from this study are similar to the reports of Auta and co-workers (2017) and Padrao *et al*. (2016).

A major structural difference between plant cellulose and BC is the microfibre network of BC. While BC exhibits a network of packed microfibrils, plant cellulose shows large bundles which are disjointed (figures 6.5b and 3.3a). Past findings revealed that crossing of the cellulose microfibrils in BC results in increase in bundles and this is due to increasing hydrogen bonds (Sheykhnazari *et al*., 2011). This is important, since it provides a means of

attachment for bacterial cells, as seen in figure 6.5a. Some researchers (Rezaee *et al*., 2008; Jagannath *et al*., 2010, Fijalkowski *et al*., 2016) have taken advantage of this property and have immobilised several bacteria on BC. The SEM micrographs from this study from both media are similar, figures 6.5a and 6.5b show the microfibrillar structure of BC obtained from MHS medium. The SEM micrograph from unwashed BC (Figure 6.5a) shows bacterial cells attached to the BC fibre network, a feature which is important for this study. Figure 6.5b shows the SEM micrograph of BC obtained from MHS medium after purification, the microfibrillar network is not affected by washing with NaOH. The micrographs obtained from this study are similar to the observations of Gupta *et al*. (2016) who reported that BC produced from *G. xylinus* ATCC 23770 is made up of ribbons of cellulose that entangle each other, thus creating a dense fibre network structure.

X- ray diffraction was used to study the crystallinity of BCs obtained from both media (figure 6.8a and 6.8b). BC sample from HS medium showed peaks at 14.5 (2θ), 23 (2θ) and at 44 (2θ), this is similar to peaks obtained from BC sample from MHS medium at 13 (2θ), 22 (2θ) and at 44 (2θ). These well- defined peaks at 14.5 (2θ), 23 (2θ) (HS medium BC), 13 (2θ) and 23 (2θ) (MHS medium BC) show that cellulose obtained during this study is the type-1 cellulose according to the reports of Auta *et al*. (2017) and Kwak *et al*. (2015). Past studies have shown that high intensity diffraction peaks at 14.5 (2θ) and 22.6 (2θ) are indicatives of type-1 cellulose (Sheykhnazari *et al*., 2011).

During the study of the production of BC from HS and MHS media, it was discovered that the presence of citric acid in the culture medium had no effect on BC production rather a higher yield was recorded in the absence of citric acid in MHS medium. It was also discovered that there were no differences in the structure, physical and chemical characteristics of BCs obtained from both media. There was no physical difference between

the wet and dried BCs from both media, they showed similarities in the arrangement of their microfibrils when examined using SEM, similar FT-IR peaks were obtained as well as similar peaks in their crystallinity and the type of cellulose (Type-1) obtained from the media.

Hence, MHS medium was selected for further studies during this work due to the high yield (W_w 78.88g/l and W_d 1.37g/l) recorded.

Dried BC sheets obtained from MHS medium were milled using the pulverisette 14 (Fritsch, Germany) fitted with 0.08mm sieve ring size. Milling was performed for the ease of working with a powdered substance and to increase the surface area. Obtained powdered BC (PBC) was analysed to investigate if the shearing force of the rotor blades had any detrimental effect on the chemical structure of BC.

FT-IR, SEM and XRD were carried out to analyse the effect of milling on BC (Figures 6.7, 6.8 and 6.9). The analyses carried out revealed that there was no detrimental effect on the structure and characteristics of BC as a result of the shearing forces of the rotor blade. SEM analysis shows that the microfibrils of PBC are tightly packed together compared with BC. FT-IR spectra revealed peaks that are similar to the ones obtained from dried BC sheets while XRD showed peaks corresponding to cellulose type-1 at 22 (2θ).

8.5 Comparing the Effect of Known Cryoprotectants with BC, PBC and γ-PGA on Probiotic Strains on Bifidobacteria during Freeze-Drying

Freeze drying has been reported to be one of the best methods of preserving probiotic bacteria due to its ability to provide milder conditions that enhances cell viability (Makinen *et al*., 2012). However, loss in viability of probiotic bacteria during freeze drying can occur due to initial concentrations, cell size (Otero *et al*., 2007), osmotic shock and membrane injury due to recrystallization and intracellular ice formation (Saarela *et al*., 2006). Freeze drying is

a common way of preserving microorganisms, since excellent long-term viability (up to 35 years in some cases) has been reported (Miyamoto-Shinohara *et al*., 2000). During freeze drying, frozen water is removed by sublimation, which reduces damages to biological structure (Capela *et al*., 2006).

Probiotic cultures for food applications are frequently supplied in frozen or dried form, as either freeze-dried or spray-dried powders (Anal and Singh, 2007). The powdered form is usually achieved through freeze drying, which has been mentioned as one of the best ways of maintaining viability of probiotic organisms (Saarela *et al*., 2006). However, loss of viability is still recorded due to reasons mentioned earlier (section 1.5.1).

Researchers have used different cryoprotectants to reduce viability loss during this important stage of probiotic preparation, some of the commonly used cryoprotectants include sucrose or skimmed milk powder, these have been used for different microbes (Hubalek *et al*., 2003) as well as for probiotic bacteria. Other commonly used cryoprotectants include fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) (Shokri *et al*., 2015) or lactose (Vinderola *et al*., 2011). Different support systems, such as alginate (Martin *et al*., 2015), whey protein isolate microbeads (Doherty *et al*., 2012), a mixture of sodium caseinate and gellan gum (Nag *et al*., 2011), etc., have been investigated for their potential ability to immobilize and act as cryoprotectants for probiotic bacteria.

Due to the ultrafine network structure and the fact that the producing bacterium *G. xylinus* ATCC 23770 can attach itself to the fibre network of BC (as evidenced from our SEM micrograph Figure 6.5a), the immobilization potential of BC with denitrifying bacteria was investigated by Rezaee *et al*., (2008). Immobilising the bacteria with BC increased the adsorption capacity, and decreased cell leakage from the beads. To the best of our knowledge, the studies available on the use of BC as an immobilization support system for

probiotic bacteria are the reports of Jagannath and co-workers (2010) and Fijalkowski *et al*. (2016). The investigation of Fijalkowski *et al*. (2016) was focused on investigating the protective effect of BC on probiotics in simulated gastric juice and bile salts.

Jaganath *et al*. (2010) investigated the immobilization potential of BC using lactobacilli, they reported that the fibrous structure of BC functioned as a physical barrier against the deleterious effect of freezing and that BC also provided an attachment matrix for the *Lactobacillus* species investigated.

During this study, sterile wet BC sheets were used as immobilization support systems for *B. longum* NCIMB 8809 and *B. breve* NCIMB 8807 during freeze drying while the cryoprotective effect of PBC and γ-PGA were compared with SMP and sucrose in six bifidobacteria strains.

When sterile wet BC sheets were used as protective support for *B. longum* NCIMB 8809 and *B. breve* NCIMB 8807 during freeze drying. An average viable count of 10^9 CFU/ml was recorded in both strains before the addition of sterile BC sheets. It was challenging assessing the viability of probiotic cells after freeze drying as cells were not dislodged from the cellulose fibre network which is contradictory to past report where lactobacilli species were immobilised on BC sheets before freeze drying with an initial count of 10^9-10^{10} CFU/g. After freeze drying, samples were brought back to original volume using physiological saline, approximately 2 Log CFU/ml reduction was recorded immediately after freeze drying in BC-immobilised cells (Jagannath *et al*., 2010).

SEM was then used to investigate the attachment of probiotic cells to the network fibre of BC. SEM analyses revealed the attachment of probiotic *B. breve* NCIMB 8807 to the ultrafine fibre network of BC (Figures 7.2b). The SEM micrograph of the control sample (Figure 7.2c) which contained cut cubes of BC inoculated in BSM broth with no probiotic

bacteria, revealed the ultrafine fibre network of BC. The SEM result was similar to the result of Jagannath *et al*. (2010) where the SEM micrograph of lactobacilli immobilised on BC sheets showed cells attached to the BC fibres.

In this study, the cryoprotective effect of γ-PGA and PBC was compared with the cryoprotective effect of skimmed milk powder (SMP) and sucrose on six bifidobacteria strains. SMP and sucrose are well known cryoprotectants and they have been used in previous studies (Gisela *et al*., 2014; Shu *et al*., 2015) for protecting different probiotic bacteria during freeze drying and approximately 65% probiotic survival was reported by Gisela and co-workers (2014).

An initial average cell count of $\approx 10^9$ CFU/ml was recorded in all strains excluding *B. bifidum* NCIMB 702715, where an initial count of 10^6 CFU/ml was recorded before freeze drying (Figures 7.1a – 7.1f). After freeze drying, ≈2 Log CFU/ml and ≈ 3 Log CFU/ml reductions were recorded in the control samples and PBC protected samples respectively. In γ-PGA protected samples, ≈ 1 Log CFU/ml reduction was recorded in all strains whilst sucrose and SMP protected cells showed ≤1 Log CF/ml reduction after freeze drying.

Sucrose and SMP are known cryoprotectants and it is not surprising that they have the best protective ability from this study. However, the cryoprotective effect of γ-PGA is comparable to the cryoprotective effect of SMP and sucrose, in fact, γ-PGA was a better cryoprotectant for *B. infantis* NCIMB 702255 as observed from our study (Table 7.1). In *B. bifidum* NCIMB 702715, PBC was a better cryoprotectant than γ-PGA and SMP, and sucrose was the best cryoprotectant.

The results obtained in this study correspond with results from past findings, where sucrose and skimmed milk have shown to have very good cryoprotective abilities. Jagannath *et al*. (2010) compared the cryoprotective effect of wet BC sheet (nata), PBC and skimmed milk on

lactobacilli species. When cells were protected with PBC, a 4 Log loss was recorded after freeze drying while a 2 Log reduction was recorded in BC sheet and skimmed milk, in our studies, PBC demonstrated the lowest loss in viable cells after freeze drying compared with the other cryoprotectants except for application with *B. bifidum* NCIMB 702715. Siaterlis and co-workers (2009) also investigated the ability of different concentrations of trehalose, sorbitol or sucrose to protect lactobacilli species during freeze drying. Out of the three sugars examined, sucrose provided better protection during freeze drying compared with sorbitol and trehalose.

Finally, SEM was used to investigate how the probiotic cells were protected by the different cryoprotectants. In the control samples (figure 7.3a), cells were observed to be clustered around each other with no form of coating or covering, in PBC (figure 7.3c), cells were observed to be clustered around the cellulose fibres, in γ-PGA (Figure 7.3e), cells were embedded within the γ-PGA matrix and cells were also covered with a thin layer of γ-PGA, while in SMP (Figure 7.3f), cells were seen on the surface of grainy SMP and cells appear to be covered with a layer of SMP. It was difficult analysing sucrose protected cells due to the nature of freeze dried sucrose as it begins to absorb moisture content once samples were taken off the freeze drier. Report of the SEM analyses is similar to the reports of Bhat *et al*. (2013) who assessed the potential of γ-PGA as a cryoprotectant for probiotic bacteria (lactobacilli and bifidobacteria) and compared it with sucrose. The cryoprotective ability of γ-PGA and sucrose were comparable in *Bifidobacterium* species and the SEM micrographs of probiotic cells protected with γ-PGA revealed that the cells were embedded within γ-PGA matrix. Jagannath *et al*. (2010) who evaluated the cryoprotective abilities of different cryoprotectants including sucrose, BC and PBC reported that *L. debrueckii* cells were immobilised in between BC fibres and in the matrix of SMP.

That γ-PGA is very promising as a cryoprotectant can be seen from our results and this corresponds with past findings. Bhat *et al*. (2013) investigated the cryoprotective ability of 10% γ-PGA on *L. paracasei*, *B. longum* and *B. breve*, and compared with sucrose. It was reported that 10% γ-PGA protected *L. paracasei* better than 10% sucrose, whilst their cryoprotective abilities with *B. longum* and *B. breve* were comparable.

Overall, SMP and sucrose (Table 7.1) are more effective in protecting the investigated bifidobacteria strains during freeze drying. γ-PGA is also promising since viability was maintained effectively. PBC appears promising since the loss in viability (≈2 Log CFU/ml loss) recorded during this study is better than the loss of viability (4 Log CFU/ml) recorded after freeze drying when lactobacilli species were protected with PBC (Jagannath *et al*., 2010). PBC can be incorporated along with other protective agents, as reports have shown that using a mix of different protective agents provides better protective effect than using a single protective agent (Qing-Quing *et al*., 2012).

Finally, it is important to note that probiotic product encapsulated with SMP and sucrose will not be suitable for individuals with health conditions such as lactose intolerance and diabetes, this makes γ-PGA and PBC a better choice as a cryoprotectant for individuals with health needs.

8.6 Antimicrobial Properties of Bifidobacteria Strains on Selected Pathogenic Bacteria

Antimicrobial activity is one of the diverse influences probiotic bacteria have on the host. As mentioned in the introduction (See section 1.4), the antimicrobial activity of probiotic bacteria is exerted through one of the following ways: reduction of luminal pH by the production of organic acids, secretion of antimicrobial substances such as bacteriocin, inhibition of bacterial invasion and blocking other bacteria from adhering to epithelial cells

and stimulation of the host's immunity (de Roos and Katan, 2000; Cheikhyoussef *et al*., 2007; Ng *et al*., 2009; Oelshlaeger, 2010).

During this study, selected bifidobacteria strains were investigated for their antimicrobial activities against four pathogenic bacteria; Gram positive *Listeria innocua* NCTC 11288 and *Staphylococcus aureus* NCIMB 6571, and Gram negative *Salmonella typhimurium* WLV 73 Cardiff Collection and *Escherichia coli* W1485-K12 W-T Cardiff Collection. The well-diffusion assay technique was used for this investigation (section 4.3.6.1 and 4.3.6.2). The antimicrobial properties of non-pH adjusted cell free culture supernatants (CFCS) and pH adjusted CFCS (concentrated and non-concentrated) of selected bifidobacteria against the selected pathogens were investigated.

The CFCS was analysed to confirm that there were no cells present in the CFCS. The initial pH was 6.50 in all probiotic culture media, a reduction in pH within the range of 5.97 - 4.33 (see table 7.1) was recorded in all bifidobacteria strains under investigation, which was similar to the result of Tejero-Sarinena *et al*. (2012) who recorded pH between pH 5.90 – pH 3.60 in the CFCS of probiotic strains investigated. Lievin *et al*. (2000) also observed similar trend (pH 4 – pH 4.5) in the change in pH of the CFCS during the investigation of the antimicrobial activities of bifidobacteria strains isolated from infant stools. Likotrafiti *et al*. (2013) also reported a pH range of pH 4 – pH 4.5 in the CFCS of *L. fermentum* and *B. longum* during the investigation of their antimicrobial activities against enterohaemorrhagic *E. coli* and enteropathogenic *E. coli*.

The pH adjusted CFCS in this study when in the non-concentrated state showed no inhibition against the selected pathogens (table 7.3), however in the concentrated state, varying degrees of inhibition were observed against the growth of the pathogens.

In the non-adjusted pH CFCS (table 7.2), *B. adolescentis* NCIMB 702229 demonstrated the least inhibitory effect against all the investigated pathogens both in the concentrated and non-concentrated state. There was no inhibition when the non-concentrated CFCS of *B. infantis* NCIMB 702255 was investigated for its inhibitory effect on the growth of the pathogens. However, inhibition was recorded in *L. innocua* NCTC 11288, *E. coli* W1485-K12 W-T and *S. typhimurium* WLV 73 Cardiff Collection in the concentrated state.

In the non-adjusted pH CFCS (table 7.2), *B. longum* NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 showed the best inhibitory effects against the selected pathogens as they all demonstrated inhibition against all the investigated pathogens both in the concentrated and the non-concentrated state. In the concentrated state, CFCS of *B. longum* NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 showed greater antimicrobial properties against *L. innocua* NCTC 11288 and *S. typhimurium* WLV 73 Cardiff Collection compared with the non-concentrated CFCS. The inhibitory effects of *B. longum* NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 in the non-concentrated state was higher in *S. aureus* NCIMB 6571 compared with the concentrated state, while in *E. coli* W1485-K12 W-T, *B. animalis* NCIMB 702716 showed a greater inhibitory effect in the concentrated state while *B. longum* NCIMB 8809 and *B. breve* NCIMB 8807 showed greater inhibitory effect in the non-concentrated state. CFCS of *B. bifidum* NCIMB 702715 demonstrated inhibitory effect against all the pathogens (Table 7.2) except *S. aureus* NCIMB 6571, however, the concentrated CFCS from *B. bifidum* NCIMB 702715 showed inhibitory effect against *S. aureus* NCIMB 6571.

Anand *et al*. (1981), investigated the antagonistic effect of six *B. bifidum* strains against the growth of pathogens including *S. aureus, E. coli* and *S. typhosa*. The pH of the CFCS of *B. bifidum* strains were adjusted to different pH values between 5.0 - 7.0, the pH of the untreated

CFCS was 4.5. CFCS showed antibacterial activity from pH 4.50 - pH 5.0, at pH 4.50, an 18mm inhibitory zone was reported against *E. coli*, whilst at pH 5.50, a 17mm inhibitory zone was reported. This is similar to our report where 11.3mm zone of inhibition was reported against *E. coli* W1485-K12 W-T and the pH of the CFCS of *B. bifidum* NCIM 702715 was 5.97. The different strains of *B. bifidum* also showed antibacterial activities against *S. typhosa* and *S. aureus*, in our study, *B. bifidum* NCIMB 702715 showed inhibitory effects against *S. typhimurium* WLV 73 Cardiff Collection and *S. aureus* NCIMB 6571.

Tejero- Sarinena *et al*. (2012) investigated the antimicrobial properties of selected probiotic strains including *B. longum*, *B. breve*, *B. bifidum* and *B. infantis* and some lactobacilli strains on different Gram negative and Gram positive pathogens. When the agar spot test assay was used to investigate the inhibitory effects of these probiotic strains, ≤5mm - ≥17mm zones of inhibition were reported in the bifidobacteria strains. *B. infantis* showed the least inhibitory effect of all the investigated bifidobacteria strains and no inhibitory effect was recorded at 24 hours with *B. infantis*. The well diffusion assay was then carried out on the best strains from the agar spot test which included *B. breve*, *B. longum* and *B. bifidum*. There was no inhibitory effect on selected pathogens when the pH adjusted CFCS of the different probiotic strains were used. However, when the concentrated pH adjusted CFCS were investigated, it was reported that the inhibition observed was a zone of yellow colouration around the wells.

This is similar to our reports, where no antimicrobial property was recorded at non-concentrated pH 7 CFCS, but antimicrobial property was recorded in three (*B. longum* NCIMB 8809, *B. adolescentis* NCIMB 702229 and *B. infantis* NCIMB 702255) when pH 7 CFCS was concentrated. In the non-adjusted pH CFCS, our reports show that the CFCS of most of the probiotic strains produced inhibitory effects against the growth of *S. aureus* NCIMB 6571 when concentrated and not concentrated. This is contradictory to the report of

(Tejero-Sarinena, 2012) who reported no inhibition against the growth of *S. aureus* with the non-concentrated CFCS of all the probiotic strains, but inhibition was recorded when concentrated.

In the report of Fooks and Gibson (2002), where the inhibitory effects of some lactobacilli strains and *B. bifidum* fermented with different carbohydrate sources were investigated, the neutralised supernatant showed lesser inhibitory effect compared with non-pH adjusted supernatant. Our report is similar to reports from these studies where lesser inhibitory effects are recorded in pH adjusted CFCS. Likotrafiti *et al*. (2013) also reported that neutralized supernatant resulted in lesser inhibitory effects in selected probiotic strains.

Furthermore, there seems to be a correlation between pH and inhibitory effects as evidenced from our results. There was no inhibition of the pathogens at pH 7 in the non-concentrated state (although inhibition was recorded in the concentrated state), while inhibition was recorded in the untreated CFCS with pH range of 5.97-4.33. The three strains with the lowest pH *B. longum* NCIMB 8809 (4.47), *B. breve* NCIMB 8807 (4.33) and *B. animalis* NCIMB 702716 (4.58) showed greater inhibition compared with the other strains. This was similar to the reports of Likotrafiti *et al*. (2013) and Tejero-Sarinena *et al*. (2012), who reported that the lower the pH of the CFCS, the higher the inhibitory effects exerted by the investigated probiotic strains. The highest inhibitory effect was also recorded at a lower pH of 4.2 during the investigation of antimicrobial activities of bifidobacteria strains (Trejo *et al*., 2006).

According to Fooks and Gibson (2001), some findings have contradicted the finding that inhibition is due to low pH. Therefore, reduction in pH may not be the major inhibitory parameter. In addition, different reports have suggested that there are other mechanisms apart from reduction in pH that are responsible for the inhibitory effects of bifidobacteria strains. These include the ability of probiotic strains to ferment carbohydrates, thereby producing

organic acids, and ability to produce other antimicrobial compounds such as hydrogen peroxide, strain specific metabolites, non-lactic acid molecules, biosurfactants or bacteriocins (Servin, 2004; Fayol-Messaoudi *et al*., 2005; Tejero-Sarinena 2012).

In addition, as discussed in section 1.4 that probiotics can exert their antipathogenic effects through five means, one of which includes degradation of toxins and toxin receptors. *S. aureus* is found on the skin and in the nose of about 25% healthy adults, however it can intoxicate foods and produce one or more preformed enterotoxins that can cause food poisoning (CDC, 2016; Argudin *et al*., 2010). The other three pathogens investigated colonise the digestive tract and cause infections. Therefore, the mode of action of inhibition for *S. aureus* in an *in vivo* situation will be through the degradation of the consumed toxin or toxin receptors whilst the mode of action against the other three pathogens under investigation can be through any of the other means described in section 1.4. This could be a possible explanation for the reduced inhibitory property of the CFCS against *S. aureus*. In the concentrated and non-concentrated state of the untreated CFCS, four out of the probiotic strains had an inhibitory effect against *S. aureus* while in the pH adjusted CFCS, three out of the selected probiotic strains demonstrated inhibition against *S. aureus* in the concentrated state and no inhibition was recorded in the non-concentrated state. The inhibitory property demonstrated against *S. aureus* could be because of reduction in pH.

Finally, results from this study further confirm that the benefits associated with probiotics are strain specific and not species specific (Bevilacqua *et al*., 2003; Figuerora-Gonz-alez *et al*., 2011). This was also confirmed by the study of Jacobsen and co-workers (1999) during the investigation of the antimicrobial activities of 47 strains of lactobacilli against pathogens. It was reported that the antimicrobial activities of the strains varied, while some showed broad

inhibitory effects, others showed weak inhibitory effects while some showed no inhibitory effects against the pathogens.

8.7 Antimicrobial Activity of Organic Acid Production

Production of organic acids by some bifidobacteria strains is responsible for the reduction of pH in culture medium during fermentation (Makras and Vuyst, 2006). Lactic acid and acetic acid are mostly produced during the fermentation of bifidobacteria strains, although there are reports of production of other organic acids such as propionic acid and formic acid (Trejo *et al.*, 2006; Likotrafiti *et al*., 2013).

The inhibitory effects of bifidobacteria strains towards Gram positive and Gram negative pathogens have been linked with reduction in pH due to organic acid production, inhibitory actions of undissociated organic acid molecules, competition for nutrients and production of specific antibacterial substances (Cheikhyoussef *et al*., 2008).

During the organic acid assay experiment, the growth of probiotic organisms, the pH, and concentration of organic acid produced was determined at intervals (0hr, 8hrs, 24hrs and 48hrs). The growth of bifidobacteria is enhanced by culture media and high growth rate has been recorded in nutrient rich medium such as trypticase phytone yeast extract (TPY) and De Man Rogosa and Sharpe (MRS) media (Martinez *et al*., 2013).

Biedrzycka *et al*. (2003) reported the presence of lactic acid and acetic acid as the main product of fermentation of bifidobacteria strains, however, a higher concentration of lactic acid was produced in culture media containing lactose and glucose as substrates. In this study, TPY contains glucose as substrate (see table 4.5), hence concentration of lactic acid and acetic acid were investigated in this study.

Acetic acid was produced sparingly by the investigated probiotic strains. At 24 hours, maximum acetic acid concentration (0.13g/l) was detected in *B. longum* NCIMB 8809 while at 48 hours, maximum acetic concentration (0.14g/l) was detected in *B. breve* NCIMB 8807. The concentration of lactic acid produced during this study increased from 0.01g/l at 0 hour to 1.76g/l at 48 hours (Figure 7.4). Maximum concentration of lactic acid (1.76g/l) was detected in *B. longum* NCIMB 8809 at 48 hours, whilst reduction in pH from pH 4.26 – pH 4.17 was also recorded during this period (see section 7.2.2).

It is important not to misinterpret the organic acid result since the concentration of an acid might not determine its strength. Hence, the need to find out if the reduction in pH was due to the presence of acetic acid (even though concentration was low) or lactic acid. It is known that the stronger an acid, the larger its dissociation constant (K_a) value, but because K_a involves negative power, it is usually expressed as pK_a. Therefore, the stronger an acid, the larger its K_a and the smaller its pK_a (Morris, 1968). The pK_a values of lactic acid and acetic acid 3.08 and 4.76 (Likotrafiti *et al*., 2013) show lactic acid to have a lower pK_a value compared to acetic acid, confirms that the reduction in pH is a result of the presence of lactic acid in the culture media.

From six strains investigated, the four strains with the highest lactic acid concentrations were *B. longum* NCIMB 8809 (1.76g/l), *B. breve* NCIMB 8807 (1.70g/l), *B. bifidum* NCIMB 702715 (1.75g/l) and *B. animalis* NCIMB 702716 (1.66g/l), and these demonstrated the greatest antimicrobial properties (tables 7.2 and 7.3) against the selected pathogens with marked reductions in pH (table7.4). Likotrafiti and co-workers (2013) stated that at pH higher than the pK_a values, the acid is undissociated, which results in increased antimicrobial effect. This was also confirmed by the reports of de Keersmaecker *et al*. (2006) and Cheikhyoussef *et al*., (2008). In our study, the pH of the culture media was higher than the pK_a value of lactic

acid (3.08) all through the experiment. Therefore, it is possible to conclude that that there is a link between pathogen inhibition and lactic acid production, since *B. longum* NCIMB 8809 and *B. breve* NCIMB 8807 (strains with the highest lactic acid concentrations) demonstrated the best antimicrobial effects against the pathogens used in this study. The lactic acid concentrations of *B. bifidum* NCIMB 702715 and *B. animalis* NCIMB 702716 were the same (1.52g/l) at 24 hours, although *B. animalis* NCIMB 702715 showed better inhibitory effects when compared with *B. bifidum* NCIMB 702715. *B. adolescentis* NCIMB 702229, with the lowest lactic acid concentration (0.56g/l), showed the least inhibitory effect against the pathogens, which further links lactic acid production with inhibitory effect.

This result is in accord with the report of Makras *et al*. (2005), who found that lactic acid is the major metabolic end product of *L. paracasei* subsp. *paracasei* 8700:2, although other metabolic products such as acetic acid, ethanol and formic acid were reported to be produced as well. Tejero-Sarinena *et al*. (2012) also reported a high concentration of lactic acid compared with acetic acid in all the putative probiotics (lactobacilli and bifidobacteria) investigated, with production of lactic acid leading to the reduction in pH, therefore resulting in an antimicrobial effect on the pathogens which were both Gram-negative and Gram-positive. They also reported that the strain with the highest amount of lactic acid and greater reduction in pH of the medium demonstrated the largest inhibition zones against all pathogen strains. It was also reported by de Keersmaecker *et al*. (2006), that upon removal of lactic acid from CFCS of *L. rhamnosus*, the antimicrobial effect was completely lost but antimicrobial effect was unaltered upon dialysis with lactic acid, which indicated that lactic acid is a major antimicrobial compound.

In our study, we investigated the antimicrobial property of commercial lactic acid to confirm whether the inhibitory effect of the probiotic strains was due to the presence of lactic acid.

Commercial lactic acid with the same concentration of lactic acid present in the CFCS of the probiotic strain was used (1.56g/l). There was no inhibition of the pathogens at this concentration, however, with 2g/l lactic acid concentration, there was inhibition of the growth of *S. typhimurium* WLV 73 Cardiff Collection and *S. aureus* NCIMB 6571, while no inhibition was recorded in *L. innocua* NCTC 11288 and *E. coli* W1485-K12 W-T. This report is similar to the findings of Collado *et al*. (2005), who found there was no antimicrobial effect upon addition of lactic acid and acetic acid against *Helicobacter pylori*, and they suggested that there are other compounds responsible for the antimicrobial properties of neutralised CFCS against this pathogen.

That there was no inhibition when the same concentration of lactic acid was tested points to the fact that there are other factors that are responsible for producing inhibitory effects against pathogens. According to Makras *et al*. (2006) who investigated the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica seriva* Typhimurium SL1344, the antibacterial activity exhibited by some of the investigated strains (*L. johnsonii* La1 and *L. plantarum* ACA-DC 287) was as a result of the production of lactic acid and other unknown substances that were only active in the presence of lactic acid. This could be a possible explanation for the result obtained when the antimicrobial activity of commercial lactic acid was tested on the selected pathogens

Furthermore, it is important to note that lactic acid is not accumulated in the colon because it can be absorbed by the host and it can be converted into short chain fatty acids (SCFAs) such as butyrate, propionate and acetate by some species such as *Eubacterium hallii* (Munoz-Tamayo *et al*., 2011; Rios-Covian *et al*., 2016). It is therefore detected in low levels in the gut contents of healthy individuals (Duncan *et al*., 2004, Munoz-Tamayo *et al*., 2011). Conversion of lactic acid into SCFAs by lactic acid utilizing bacteria in the gut is essential

since high concentrations of lactic acid have been linked with health conditions such as short bowel syndrome, ulcerative colitis, neurotoxicity and cardiac arrhythmia, whilst in ruminants, it can lead to acidosis and lameness in horses (Munoz-Tamayo *et al*., 2011). SCFAs have been reported to have immune modulatory effects, suppressing inflammatory cytokine secretion in cultured epithelial cells and also improving model colitis in mice (Neish, 2009). Since lactic acid does not accumulate in the colon, but rather converted to SCFAs this appears to be one of the links between probiotics and the health benefits they provide *in vivo*.

Finally, from our studies, we can conclude that the presence of lactic acid is responsible for the reduction in pH of the CFCS. It can also be said that the major inhibitory mechanism observed in this study is due to pH reduction which is as a result of lactic acid production, Vanderpool *et al*. (2011) reported that the reduction of local pH due to the production of lactic, acetic and propionic acids inhibits the growth of a wide range of Gram- negative pathogenic bacteria. However, this might not be the case in an *in vivo* situation since lactic acid does not accumulate in the gut but rather converted to SCFAs such as butyrate (Rios-Covian *et al*., 2016). Antimicrobial property, and lactic acid production are also strain specific and not genus specific, since the different strains produced varying concentrations of lactic acid and demonstrated different degrees of inhibition of the pathogens investigated.

8.8 Protection in Simulated Gastro Intestinal Conditions

The final aim of this research was to investigate the effect of protecting selected probiotic strains with γ-PGA, PBC or a combination of γ-PGA and PBC (γ-PGA+PBC) in simulated intestinal conditions. Three out of the six investigated probiotic strains were selected for this experiment due to their antimicrobial properties. The three strains (*B. longum* NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716) were selected because they showed better antimicrobial properties compared with the other strains investigated previously.

As has been discussed previously (see section 1.5), it is a known fact that processing and storage conditions can affect survivability of probiotics. However, physical conditions in the human gastrointestinal tract has a major influence on probiotics survival after ingestion (Pinto *et al*., 2015). The acidic pH of the stomach and the presence of bile salts and hydrolytic enzymes in the small intestines are some of the major challenges of safe delivery of probiotic strains to the target site where they colonise the epithelial lining of the large intestine (Gandomi *et al*., 2016; Prakash *et al*., 2011). Researchers have employed different means, such as incorporating into yoghurt, fermented milk or cheese and microencapsulating with sweet whey and prebiotics, to deliver probiotic strains to the target site (Makelainen, *et al*., 2009; Pinto *et al*., 2015).

The major challenge associated with using some of these carriers is the fact that they might not be suitable for all individuals. Individuals who are lactose intolerant will be unable to use probiotic products containing yoghurt or milk, whilst the presence of sugar is also detrimental to individuals who are diabetic. Hence, there is a need for the use of other carriers that will provide protection without having any negative impact on the consumers.

γ-PGA and BC are natural and edible biopolymers that can be utilised as protective agents. As previously discussed (section 2.1), γ-PGA occurs naturally in natto (Ashiuchi, 2013), whilst BC is a source of dietary fibre in nata (Jagannath *et al*., 2010). γ-PGA is known to be stable in low pH and disintegrates in neutral or weaker environments (Ho *et al*., 2006), conditions found in the GIT with a region of low pH (between 1.8 - 3.0) in the stomach and near neutral pH in the duodenum, so it is possible that it may be protective against stomach acids. BC has a unique ultra-fine network of fibres that probiotic strains can attach themselves to while transiting the GIT, so may also be able to confer a protective effect. These two biopolymers were used in this study to investigate the delivery of probiotic *B.*

longum NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 as they transit through the stomach and during exposure to bile salts in the small intestine. Researchers have investigated carriers such as alginate-milk microspheres (Shih *et al*., 2013), pectin-whey protein (Gebara *et al*., 2013) and chitosan-alginate with inulin (Gandomi *et al*., 2016) to protect *Lactobacillus* species in simulated gastrointestinal conditions. Most of these studies were performed for a maximum period of 2 hours in simulated gastric juice and most of the research have been focused on the *Lactobacillus* species.

8.8.1 Protection in Simulated Gastric Juice

The protective effect of 5% γ-PGA, 5% PBC and 5% γ-PGA+PBC (2.5% γ-PGA(w/v) and 2.5% PBC(w/v)) was investigated on *B. longum* NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 in a simulated gastric environment (SGJ). Unprotected *B. longum* NCIMB 8809 and *B. breve* NCIMB 8807 cells showed total loss in viability after 1 hour (Figures 7.5 and 7.6) whilst unprotected *B. animalis* NCIMB 702716 cells showed total loss in viability after 2 hours (Figure 7.7). However, after 4 hours of incubation in SGJ, protected cells showed a reduction of ≤0.54 Log CFU/ml, ≤2.74 Log CFU/ml and ≤0.87 Log CFU/ml (γ-PGA, PBC and γ-PGA + PBC respectively).

From the result obtained, it was important to investigate the effect of 2.5% γ-PGA and 2.5% PBC individually to know which of the two biopolymers enhanced the protective effect of the combination. When probiotic strains were protected with 2.5% γ-PGA and incubated in SGJ for 4 hours, viability after 4 hours was comparable with viability at 0 hour and there was no significant difference ($p>0.05$) (Figures 7.8, 7.9 and 7.10). However, when 2.5% PBC was used to protect the probiotic strains in SGJ, ≈4 Log CFU/ml loss was recorded after 4 hours in all three strains.

From this study, γ-PGA and γ-PGA+PBC were better at protecting the probiotic strains during exposure to the low pH of simulated gastric juice compared with PBC on its own, where ≈ 2.36 Log CF/ml loss was recorded in all strains after 4 hours of incubation in SGJ. These results also confirm the reports of Qing-Quing *et al*. (2012), where an optimised mix of different cryoprotectants provided better protection for *B. animalis* subsp. *Lactis* Qq08 during freeze drying.

Ho and co-workers (2006) reported that at pH 2.0, γ-PGA forms a tight and compact α-helix conformation, resulting in a strong hydrophobic character and making it insoluble in water. They also reported that an increase in pH caused hydrogen bonds to break and changes the conformation from α-helix to random-coil, this causes the ionisation of the α-COOH group to $\alpha\text{-COO}^-$. This is likely to be the mechanism of protection of probiotic cells by γ-PGA and γ-PGA+PBC in simulated gastric juice.

PBC provided protection for the probiotic cells to a certain extent, which could be attributed to the ultra-fine network fibre structure of PBC. However, it could be due to buffering capacity, since it has been reported that polysaccharides can act as buffers thereby, reducing the activity of acids (Fareez *et al*, 2015).

An investigation on subjecting BC films to gastrointestinal system by using it as an edible antimicrobial packaging for sausage was reported by Padrao *et al*. (2016). That study was aimed at investigating antibacterial properties of edible BC films modified by bovine lactoferrin, they also examined the generation of cytotoxic elements by modified BC films during digestion. Significant inhibition of pathogenic bacteria (*S. aureus* and *E. coli*) was reported, and the modified BC films and their simulated digestive products did not exhibit cytotoxicity against fibroblasts (Padrao *et al*., 2016). From our results and their results, there is a clear indication that BC can be used as a protective agent, which will allow probiotic

organisms to survive the stomach conditions and be able to carry out their health benefits on the host.

As far as we know, the study of Fijalkowski *et al*. (2016) is the only study that has investigated the protective properties of bacterial cellulose on probiotic strains against adverse gastric juices. In their study, wet and dry BC pellicles or BC beads obtained from static and agitated culture media respectively were used to immobilise *Lactobacillus* spp. in gastrointestinal conditions. They reported a higher number of immobilised lactobacilli in both wet forms of BC compared with dry BC pellicles, the difference between the immobilization potential of wet and the dry BC forms was attributed to the fact that wet BC demonstrates higher water absorption capacity, it is more porous than the dry BC and therefore, it will allow the cells to penetrate the deeper layers of BC. They also reported improvement in the survival of the BC immobilised lactobacilli during exposure to simulated intestinal conditions (Fijalkowski *et al*., 2016). Comparing our studies with this study show that different forms of BC (wet, dry or milled) can act as protective supports for probiotic bacteria against the adverse conditions of the gastrointestinal tract.

Different protective supports have been used in the past to support probiotics strains during transition through the GIT and most of the studies have investigated the process for about 2 - 3 hours. Cui *et al*. (2000) investigated the survival of *B. bifidum* loaded in alginate poly-l-lysine microparticles in simulated gastric fluid for 2 hours. Survival of *B. bifidum* reduced with about 2 Log CFU/ml after incubation in simulated gastric fluid. They assumed that the reason for the decline in viability was the presence of surface pinholes on the microparticles that allowed the gastric fluid to penetrate. Chavarri *et al*. (2010) also investigated the survival of microencapsulated *B. bifidum* and quercetin (a prebiotic) with alginate and a chitosan coating in simulated gastric juice for 2 hours, noting a decline in viability (< 1 Log CFU/ml)

in protected cells. The survival of *L. acidophilus* encapsulated with pectin microparticles covered with whey protein in simulated gastric juice (pH 3.0) for 2 hours has also been investigated and a reduction of 0.76 Log_{10} CFU/g was recorded in protected cells (Gebara *et al*., 2013).

Comparing our studies with these studies show that γ-PGA, γ-PGA + PBC and to an extent PBC are better protective agents due to the loss of viability reported (≈ 2 Log/ CFU/ml) in their studies. The incubation time (2 hours) in their studies was also shorter compared with incubation time of 4 hours in our study, in addition, protection of cells with γ-PGA, PBC or γ-PGA + PBC does not involve any complicated technology to achieve probiotic coating.

8.8.2 Protection in Simulated Intestinal Juice

The protective effect of 5% γ-PGA, 5% PBC and 5% γ-PGA + PBC was investigated on the survival of *B. breve* NCIMB 8807 when exposed to simulated intestinal juice (SIJ).

In all the samples tested including the control samples, there was no significant difference in cell viability at 0 hour and 3 hours ($p>0.05$) (Figure 7.11). Exposing *B. breve* NCIMB 8807 to simulated intestinal juice had no detrimental effect on its survival. This is not surprising, because probiotic bacteria are known to be able to survive within this pH range (Prakash *et al*., 2011).

Our results are contrary to the observations of Gebara and co-workers (2013), who investigated the survival of *L. acidophilus* encapsulated with pectin microparticles covered with whey protein in simulated intestinal juice. However, their process was a continuous one which is slightly different from the approach used in our study, they prepared intestinal juice by adding pancreatin to SGJ (containing encapsulated *L.* a*cidophilus*) and pH adjusted to 7.0, 3 Log CFU/ml reduction in viable cell count was recorded in free cells after 3 hours of

incubation in simulated intestinal juice while 1 Log CFU/ml reduction was recorded in protected cells.

8.8.3 Protection in Simulated Intestinal Juice and Bile Salts

Apart from exposure to gastric juice in the stomach, exposure to bile salts secretion is another limiting factor to survival and delivery of probiotic bacteria to their target sites in the intestines (Jimenez-Pranteda *et al*., 2012).

It was therefore important to investigate the protective effect of γ-PGA, PBC and γ-PGA + PBC on *B. longum* NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 during exposure to bile salts in simulated intestinal juice since bile salts are released in the upper part of the small intestines. Unprotected *B. longum* NCIMB 8809 showed complete loss in viability after 2 hours of exposure to bile salts while unprotected *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 cells survived in bile salts to an extent with a reduction of ≈2 Log in both strains after 3 hours.

After exposure to bile salts, all PBC protected cells showed ≤ 2 Log CFU/ml reduction in viable cells. In *B. animalis*, PBC protected cells demonstrated better survival in bile salts with a reduction of 0.88 Log CFU/ml in cell viability, this might be due to the bile tolerance of *B. animalis*. All γ-PGA and γ-PGA + PBC protected cells showed ≤ 0.84 Log CFU/ml reduction in viable cells which demonstrates γ-PGA and PBC as potential protective supports for bifidobacteria during exposure to bile salts. Our results indicate that protecting these strains with these carriers can enhance their survival in the presence of bile salts.

Our results are consistent with the findings of Jimeneze-Pratenda *et al*., (2012), who investigated the stability of *L. rhamnosus* and *L. plantarum* encapsulated in various microbial polymers in simulated intestinal conditions. They reported a 3 Log reduction in *L. rhamnosus*

and a 2 Log reduction was reported in *L. plantarum*, whilst a 1 Log loss was recorded in microencapsulated cells after exposure to bile salts.

Results from our investigations further show strain specificity. *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 when unprotected were tolerant to 1% bile salts concentration at pH 6.0 while *B. longum* NCIMB 8809 was not tolerant to bile salts, since complete loss in viability was recorded after 2 hours of exposure to bile salts. It is also important to note the effect of PBC protected *B. animalis* NCIMB 702716 cells, less than 1 Log CFU/ml reduction was recorded after 3 hours, which also demonstrates strain specificity to different coatings.

The results from this study are similar to those reported by Pedroso *et al*. (2012), who investigated the effect of microencapsulation using spray chilling on *B. lactis* and *B. acidophilus* in simulated gastrointestinal conditions. Unprotected *B. lactis* when exposed to SGJ (pH 1.8) and 0.3% bile salts in simulated intestinal fluid at pH 6.5 showed 87% survival with 1.3 Log CFU/ml reduction, while free *L. acidophilus* cells were below detection limit (10^2 CFU/g). However, protected *B. lactis* cells showed 90.7% viability and protected *L. acidophilus* cells showed 91.8% survivability.

Many reports have highlighted the effect of bile salts on the survival of probiotic strains while transiting the GIT as well as the effect of various probiotic delivery agents. Guerin *et al*. (2003) investigated *B. bifidum* RO71, a bile and low pH sensitive strain when encapsulated in a mixed gel made up of alginate, pectin and whey protein and exposed to 2% or 4% bile salts for 3 hours. Free cells showed reductions of 2.58 Log CFU/ml and 2.07 Log CFU/ml in 2% and 4% bile salts respectively, while cells immobilised in membrane coated beads showed 1.10 Log CFU/ml and 0.65 Log CFU/ml reductions in 2% and 4% bile salts respectively.

Survival of *L. bulgaricus* encapsulated in alginate-milk microspheres, during exposure to 1% or 2% bile salts was investigated and total loss in viability of cells was recorded in free cells after 2 hours of incubation at both concentrations. However, there was no mention of the final pH of bile salts solution before incubation. Protected cells showed 1.5 Log CFU/ml and 2.1 Log CFU/ml reductions in 1% and 2% respectively (Shih *et al*., 2013). Andriantsoanirina *et al.*, (2013) also reported the tolerance of *Bifidobacterium* human isolates to bile, acid and oxygen. Out of 142 strains under investigation, 24 showed moderate or weak tolerance to bile. They concluded that bile and acid tolerance showed biological variability among species and independent strains within a species. Our results are consistent with these findings that bile and acid tolerance is species and strain specific.

The strains investigated in our study were intolerant to simulated gastric juice (pH 2.0) since complete loss in viability was recorded after 2 hours of exposure to SGJ. Exposure of *B. breve* NCIMB 8807 to simulated intestinal juice (pH 6.0) showed no detrimental effect on the cells as the viable cell count of unprotected cells at 0 hour and 3 hours were comparable. *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 appear to be more tolerant to bile salts (1% w/v) than *B. longum* NCIMB 8809 since viability was maintained in unprotected *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 cells, whilst a complete loss of viability was recorded in unprotected *B. longum* NCIMB 8809 cells.

Finally, protection of probiotic bacteria with 2.5% γ-PGA, 5% γ-PGA and 5% γ-PGA+PBC was very effective in protecting the investigated strains during exposure to SGJ and bile salts. Protection with 2.5% PBC was not effective as ≈4 Log CFU/ml reduction in viable cell count was recorded in all three strains. However, 5% PBC provided protection for the probiotic strains up to an extent during exposure to SGJ and bile salts.

9.0 CONCLUSION

Probiotics have become popular due to their nutritional and therapeutic benefits, most especially protection against pathogens and improvement of the immune systems. The main challenge in the use of probiotic bacteria is the loss of viability during processing, storage and consumption due to the low pH of the stomach and secretion of hydrolytic enzymes and bile salts in the small intestines. Researchers have employed different means to overcome these challenges, but delivering probiotics to the target site is still a challenge.

This research was focused on investigating the use of 2 biopolymers: poly-gamma-glutamic acid (γ-PGA) and bacterial cellulose (BC) which have unique characteristics both individually and as a combination, for the protection of probiotics during freeze-drying and in simulated gastrointestinal conditions. The research also investigated the antimicrobial properties of selected bifidobacteria strains.

γ-PGA has become a polymer of choice for a wide range of applications due to its unique properties such as biodegradability, edibility and solubility (Kumar and Pal, 2015). Production and characterization of γ-PGA have been studied using different bacterial strains and different production media, since these are some of the factors that affect yield and properties of γ-PGA and determine its application. γ-PGA was produced from *B. subtilis* natto ATCC 15245 and *B. licheniformis* ATCC 9945a using GS medium and medium E. These have been used previously to produce γ-PGA and have been classified as safe for the production of γ-PGA for food applications (Zhang *et al*., 2012; Kedia *et al*., 2010).

Following production, the obtained polymer was identified as γ-PGA and molecular weight determined. It was also discovered that γ-PGA yield and molecular weight was dependent on bacterial strain, media components and cultivation conditions. γ-PGA obtained from the fermentation of *B. subtilis* natto ATCC 15245 in GS medium was chosen for further work due to higher polymer yield and molecular weight

Bacterial cellulose (BC), another interesting polymer with unique characteristics such as edibility, crystallinity, purity, lack of toxicity, ultra-fine network structure (Son *et al*., 2003) was investigated during this study. BC was produced from *Gluconacetobacter xylinus* ATCC 23770 in HS medium and MHS medium.

During the production of BC, a reduction in pH was recorded during cultivation in both media and this is due to the production of gluconic acid, a product of the fermentation of *G. xylinus* ATCC 23770. The presence of gluconic acid in culture medium has been linked with reduction in BC yield (Hwang *et al*., 1999). Higher yield of BC (78.88g/l_{ww} and 1.37g/l_{dw}) was produced in the absence of citric in MHS medium which points that BC production is not dependent on the presence of citric acid, however, the presence of an organic acid appears to be important since glacial acetic acid was used to adjust the pH of both media.

FT-IR confirmed the polymer obtained from both media was cellulose, XRD showed the cellulose obtained to be crystalline in nature which is typical of type-1 cellulose, whilst SEM revealed the unique ultra-fine network fibre of BC in the washed state with no cells attached to it and unwashed state with *G. xylinus* ATCC 23770 cells attached to the fibres. It was also discovered that milling the freeze-dried BC sheets had no detrimental effect on the characteristics and properties of BC.

The ability of wet BC pellicles to act as carrier supports for probiotics during freeze drying was investigated, since during BC production, *G. xylinus* ATCC 23770 cells attached themselves to the network of fibres of BC for protection and to access oxygen at the air-liquid interface of the culture medium. It was discovered that wet BC pellicles can act as immobilising support for bifidobacteria during freeze drying, this was confirmed using SEM which revealed the attachment of *B. breve* NCIMB 8807 to the BC fibres.

Investigating the cryoprotective effects of PBC, γ-PGA, SMP and sucrose on bifidobacteria during freeze drying demonstrated SMP and sucrose as better cryoprotectants, these are well known cryoprotectants, therefore it was not surprising. γ-PGA demonstrated to be a good cryoprotectant, in fact, it was the best cryoprotectant for *B. infantis* NCIMB 702255, PBC was not as effective as γ-PGA, although it still provided some level of protection which makes PBC a potential immobilising support material for probiotic bacteria.

Some of the health benefits exerted by probiotic bacteria such as improving symptoms of ulcerative colitis and Crohn's disease and the reduction of blood cholesterol require significant probiotic metabolism in the gut (Cui *et al*., 2004; Mylonaki *et al*., 2005; Miremadi *et al*., 2014), hence, will require ingestion of significant amount of probiotics, addition of cryoprotectants such as γ-PGA and BC will not only act as carrier supports but can also confer additional nutritional benefits on the host. γ-PGA and PBC as cryoprotectants can also be a means of preservation of probiotic bacteria if required in dried and powdered form.

Antimicrobial activity is one of the influences probiotic bacteria have on the host. This is known to be species and strain specific (Shokryazdan *et al*., 2014). The antimicrobial activities of the selected probiotic strains against two Gram negative (*Salmonella typhimurium* WLV 73 Cardiff Collection and *Escherichia coli* W1485-K12 W-T Cardiff Collection) and two Gram positive (*Listeria innocua* NCTC 11288, *Staphylococcus aureus* NCIMB 6571) pathogenic bacteria were investigated.

Reduction in pH (6.50 - ≈4.30) was recorded in the CFCS obtained from all probiotic strains and the strains with the lowest pH showed better antimicrobial properties. From our studies, there appears to be a link between pH reduction and inhibitory properties since the strains with the lowest pH demonstrated greatest inhibitory properties.

The presence of lactic and acetic acids in the CFCS was investigated since these have been reported to be the major fermentation products of lactic acid bacteria. The four strains (*B. longum* NCIMB 8809, *B. breve* NCIMB 8807, *B. animalis* NCIMB 702716 and *B. bifidum* NCIMB 702715) with the maximum lactic acid concentration, showed the lowest pH in the CFCS and demonstrated the best antimicrobial properties against the pathogens. It will be difficult to confirm the effect of lactic acid since it is not accumulated in the colon *in vivo* but rather converted to short chain fatty acids (Munoz-Tamayo *et al*., 2011), however, results from this study links reduction in pH due to the production of lactic acid to the antimicrobial properties exhibited by bifidobacteria.

Overall, we can confirm that inhibitory properties of probiotic strains and lactic acid production are strain specific.

Due to the low pH of the stomach, presence of hydrolytic enzymes and bile salts in the small intestine, a heavy loss in viability is recorded after the ingestion of probiotic bacteria (Fareez *et al*., 2015). γ-PGA and PBC are edible biopolymers with unique characteristics that can be used to improve survival of these gut friendly bacteria as they transit the gastrointestinal tract. γ-PGA has the ability to remain stable at low pH and to disintegrate in a weaker acid condition while the ultra-fine network structure of BC make both polymers potential carrier for probiotic bacteria (Ho *et al*., 2006; Fijalkowski *et al*., 2016).

Therefore, the selected strains (*B. longum* NCIMB 8809, *B. breve* NCIMB 8807, *B. animalis* NCIMB 702716) were protected with 5% γ-PGA, 5% PBC and 5% γ-PGA+PBC, stored in SGJ (pH 2.) for 4 hours, SIJ (pH 6.0) and in SIJ with 1% bile salts (pH 6.0) for 3 hours. In SGJ, total loss of viability was recorded at ≤ 2 hours in all unprotected cells, γ-PGA and γ-PGA+PBC protected cells showed ≤ 0.87 Log CFU/ml reduction, whilst PBC protected cells showed ≤ 2.70 Log CFU/ml reduction in viability after 4 hours. There was no detrimental

effect during storage in SIJ, however, during storage in bile salt solution, a significant reduction in viability of unprotected cells was recorded, whilst the polymers were able to improve survival of protected cells.

It is important to select agents that can protect probiotic bacteria in the key areas where loss of viability is mostly reported. γ-PGA and BC have demonstrated to be potential agents for protecting probiotic bacteria during freeze drying and during consumption, production of both biopolymers is sustainable and BC can be a source of dietary fibre (nata), these attributes make them ideal choices to protect probiotic bacteria.

In addition, probiotic bacteria are usually delivered through dairy products and as capsules, and recently in orange juice. Since both biopolymers do not affect the taste of food, they can be added to food products either individually or in combination which is a novel application. Addition of γ-PGA immobilised bifidobacteria into orange juice has been investigated and viability of bifidobacteria was improved without spoilage of the fruit juice (Bhat *et al*., 2015; Gomaa *et al*., 2016). Wet BC in its different edible forms such as nata de coco and nata de pina can be used on its own to deliver probiotics, this will be an added advantage for individuals who are lactose intolerant or who are diabetic. Probiotic immobilised wet BC can also be incorporated into food products such as yoghurt and yoghurt drinks. PBC can be used in addition with γ-PGA as immobilising agents for probiotic bacteria and incorporated into food products.

Finally, it can be concluded from this study that BC production is not dependent on the presence of citric acid. That in *in vitro* conditions, antimicrobial properties of bifidobacteria can be linked with reduction in pH due to lactic acid production and that antimicrobial properties of bifidobacteria is strain specific. BC and γ-PGA demonstrated to be potential and sustainable immobilising supports for bifidobacteria during freeze drying and in simulated

gastrointestinal conditions. Therefore, BC and γ-PGA can protect bifidobacteria from the detrimental effects of freeze drying, the harsh conditions of the stomach and exposure to bile salts and deliver them to the intestine.

10.0 FUTURE WORK

Although a lot of studies have been carried out in this field, new opportunities for future research have been identified from the results obtained from this study.

The two *Bacillus* species investigated were cultivated at 37°C for 96 hours to produce γ-PGA and maximum yields of 14.11g/l and 24.15g/l were obtained in shake flasks and in the fermenter respectively. It would be interesting to investigate if production time can be reduced to compensate for the yield so that γ-PGA production can be more cost and time effective.

It was observed during our studies that production of γ-PGA in GS medium with *B. subtilis* natto ATCC 15245 resulted in a higher γ-PGA yield than when cultivated in medium E, this could either be due to the presence of vitamin solution in GS medium or high concentration of glycerol in medium E, reports have shown that high glycerol concentration can inhibit cell growth and another report suggested glycerol should be added later during fermentation (Wu *et al*., 2010). It will be important to investigate if adding glycerol later during fermentation could result in a better yield of γ-PGA from medium E since high molecular weight γ-PGA were reportedly produced using this method (Wu *et al*., 2010). This will be very important for other applications such as waste water treatment where high molecular weight γ-PGA are required.

BC was produced from *G. xylinus* ATCC 23770 in HS and MHS media. The time taken for BC production is an important limitation to cost effectiveness. Consequently, production time needs to be reduced to improve cost effectiveness. It will be necessary to investigate means of improving BC production time through the use of continuous fermentation as reported by Ruka *et al*. (2012), where maximum BC yield was recorded on day 14 of fermentation followed by a plateau phase during which production reduced. They also proposed that gentle shaking of the fermentation flasks every five days will allow the formed pellicle to sink,

dislodge cells from pellicles, thereby allowing the cells easier access to oxygen and consequently resulting in higher BC yield.

Production of BC in this study required substrates such as glucose, yeast extract and bactopeptone which is not exactly cost effective as BC yield is low compared with the cost of production. Preliminary study of BC production using apple waste as substrates has been carried out in our lab and BC was recovered from this experiment. It will be important to follow this up because this will be a very good means of reducing production cost, as well as looking into other waste materials as nutrient sources for BC production.

Viability was measured immediately after freeze drying the protected probiotic cells. However, storing the freeze-dried samples at different temperatures (4°C-45°C) over a longer period of time (1-3 months) will be necessary to understand how the use of BC or γ-PGA can affect the viability of probiotic bacteria during normal storage procedures.

B. longum NCIMB 8809, *B. breve* NCIMB 8807 and *B. animalis* NCIMB 702716 demonstrated to have better antimicrobial properties than *B. bifidum* NCIMB 702715. Although this strain also demonstrated good antimicrobial property, the fact that it wasn't as effective as the other strains could be due to the fact that it reached its maximum cell count faster than the other strains. It will be necessary to investigate whether better antimicrobial activity of *B. bifidum* NCIMB 702715 occurs during early culture of this organism.

Reduction in pH of the cell free culture supernatant and lactic acid production were suggested to be responsible for antimicrobial property exhibited by the investigated strains. However, lactic acid does not accumulate in the normal healthy gut, hence it will be important to study the link between production of short chain fatty acids from lactic acid and also link it with antimicrobial properties of probiotic strains. It will also be important to investigate the antimicrobial properties of the short chain acids produced from lactic acid.

The simulated gastrointestinal conditions experiments showed γ-PGA and γ-PGA+PBC to be better than PBC in protecting probiotic cells during exposure to SGJ and SIJ with 1% bile salts. The experiments were carried out independently during this study. It will be important to devise an *in vitro* method that will simulate passage from stomach to duodenum, for example, through the use of linked chemostats such as the TIM-1 and TIM-2 gastrointestinal systems. These systems are multi-compartmental, dynamic, computer-controlled chemostats that simulate the upper human gastrointestinal tract and the colon respectively (Cordonnier *et al*., 2015; Venema, 2015). This will give a clearer picture of what happens to the probiotic cells during transit through the GIT.

The sodium form of γ-PGA (Na-PGA) was used for this study, it would be interesting to find out if the calcium form of γ-PGA can also be effective in delivery of probiotics through the GIT as reports (Luo *et al*., 2016) have shown that γ-PGA can increase calcium absorption in the intestine resulting in reduced bone loss. This could be an additional benefit as this will provide extra source of calcium as well as deliver probiotics to the target site.

In addition, it will be interesting to investigate the immobilisation of probiotic bacteria in wet BC using the commonly marketed nata products such as nata de coco, nata de pina which are already available in the market, this will be a potential alternative for the currently available products such as some yoghurts and tablets. Addition of these probiotic immobilised nata to food products such as yoghurts will also be an interesting area of research.

Finally, it will be important to investigate the incorporation of the novel combination of γ-PGA and PBC into food products to investigate the survivability of probiotic strains over a period of time.

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12.0 APPENDICES

12.1 One-way ANOVA result showing the effect of culture media and bacterial strain on γ-PGA production

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
B. subtilis natto ATCC 15245 in GS medium vs *B. subtilis* natto ATCC 15245 in medium E	8.077	7.615 to 8.538	Yes	****	<0.0001
B. subtilis natto ATCC 15245 in GS in medium vs *B. licheniformis* ATCC 9945a in GS medium	3.057	2.95 to 3.518	Yes	****	<0.0001
B. subtilis natto ATCC 15245 in medium E vs. *B. licheniformis* ATCC 9945a in GS medium	3.84	3.379 to 4.301	Yes	****	<0.0001
B. subtilis natto ATCC 15245 in medium E vs. *B. licheniformis* ATCC 9945a in medium E	-5.02	-5.481 to -4.559	Yes	****	<0.001
B. subtilis natto ATCC 15245 in medium E vs. *B. licheniformis* ATCC 9945a in medium E	-4.237	-4.698 to -3.775	Yes	****	<0.001
B. licheniformis ATCC 9945a in GS medium vs. *B. licheniformis* ATCC 9945a in medium E	0.7833	0.3219 to 1.245	Yes	**	<0.0027

12.2 One-way ANOVA result showing the effect of protecting probiotic strains with 5% γ-PGA in simulated gastric juice

Bifidobacterium	**Tukey's multiple comparisons test**	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
B. longum NCIMB 8809	0 hr vs. 4 hrs	0.27	0.1307 to 0.4093	Yes	***	0.0006
B. breve NCIMB 8807	0 hr vs. 4 hrs	0.2933	-0.01029 to 0.597	No	ns	0.0594
B. animalis NCIMB 702716	0 hr vs. 4 hrs	0.54	0.112 to 0.968	Yes	*	0.0132

12.3 One-way ANOVA result showing the effect of protecting probiotic strains with 5% PBC in simulated gastric juice

Bifidobacterium	**Tukey's multiple comparisons test**	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
B. longum NCIMB 8809	0 hr vs. 4 hrs	2.49	2.075 to 2.905	Yes	****	<0.0001
B. breve NCIMB 8807	0 hr vs. 4 hrs	1.853	1.11 to 2.597	Yes	****	<0.0001
B. animalis NCIMB 702716	0 hr vs. 4 hrs	2.747	2.477 to 3.016	Yes	****	<0.0001

12.4 One-way ANOVA result showing the effect of protecting probiotic strains with 5% γ-PGA + PBC (w/v) in simulated gastric juice

Bifidobacterium	**Tukey's multiple comparisons test**	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
B. longum NCIMB 8809	0 hr vs. 4 hrs	0.4733	-0.3301 to 1.277	No	ns	0.3583
B. breve NCIMB 8807	0 hr vs. 4 hrs	0.2467	-0.2152 to 0.7086	No	ns	0.4453
B. animalis NCIMB 702716	0 hr vs. 4 hrs	0.87	0.4295 to 1.311	Yes	***	0.0005

12.5 One- way ANOVA result showing the effect of protecting probiotic strains with 5% γ-PGA in simulated intestinal juice with 1% bile salts

Bifidobacterium	**Tukey's multiple comparisons test**	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
B. longum NCIMB 8809	0hr vs. 3 hrs	0.05667	-0.5189 to 0.6322	No	ns	0.9883
B. breve NCIMB 8807	0hr vs. 3 hrs	0.8433	0.1062 to 1.58	Yes	*	0.0263
B. animalis NCIMB 702716	0hr vs. 3 hrs	0.1267	-0.1913 to 0.4447	No	ns	0.6013

12.6 One- way ANOVA result showing the effect of protecting probiotic strains with 5% PBC in simulated intestinal juice with 1% bile salts

Bifidobacterium	**Tukey's multiple comparisons test**	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
B. longum NCIMB 8809	0hr vs. 3 hrs	1.313	0.3886 to 2.238	Yes	**	0.0081
B. breve NCIMB 8807	0hr vs. 3 hrs	1.16	0.4352 to 1.885	Yes	**	0.0040
B. animalis NCIMB 702716	0hr vs. 3 hrs	0.8833	0.6528 to 1.114	Yes	****	<0.0001

12.7 One- way ANOVA result showing the effect of protecting probiotic strains with 5% γ-PGA + PBC (w/v) in simulated intestinal juice with 1% bile salts

Bifidobacterium	**Tukey's multiple comparisons test**	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
B. longum NCIMB 8809	0hr vs. 3 hrs	-0.2367	-0.6604 to 0.1871	No	ns	0.3445
B. breve NCIMB 8807	0hr vs. 3 hrs	-0.1867	-0.7871 to 0.4137	No	ns	0.7562
B. animalis NCIMB 702716	0hr vs. 3 hrs	0.8633	0.2312 to 1.495	Yes	*	0.0102

List of Conference Papers

Adebayo, O., Bhat, A., Bartlett, T. and Radecka, I. (2014). Poly-gamma-glutamic acid as a cryoprtoectant for probiotics. *International Scientific Association of Probiotics and Prebiotics.* Students and Fellows Association. Annual Conference, p18

Adebayo O., Hill D., Radecka I. and Bartlett T. (2015). Production of poly-gamma-glutamic acid and its application as a cryoprotectant for probiotics. *International seminar on Biodegradable Polymers*, University of Wolverhampton.

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Adebayo O., Hill D., Radecka I. and Bartlett T. (2017). Bacterial Cellulose: A potential protective support for probiotics in simulated intestinal conditions. *Microbiology Society* Annual Conference, p421

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